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ACCUMULATION AND FUNCTION OF PROLINE IN POLLEN

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PROEFSCHRIFT

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TO MY MOTHERLAND

TO YI-QIN, FAN AND HUI

CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

Since the early 1950's, when Bathurst (1954) reported that proline is the most abundant of the free amino acids in the pollen of several grass species, many investigations have been conducted on the significance of this accumulation. It has been shown that free proline accumulates in the pollen of many species (Virtanen & Kari, 1955; Tupý, 1963; Linskens & Schrauwen, 1969). Britikov and Musatova (1964) studied the content of free proline in the pollen of about 200 species belonging to 63 families of 42 orders of angiosperms and gymnosperms. Almost without exception, the concentration of free proline in the pollen is much higher than in pistil tissues. Because this is such a general phenomenon, it is of interest to study the mechanism of proline accumulation during anther development and to investigate the function of the proline accumulated in pollen.

In the only study on proline uptake in anthers (Britikov & Musatova, 1964) it was shown that isolated buds take up proline from a proline containing solution. It is not known whether this uptake reflects the *in vivo* situation. Also, several other questions remain unanswered. What is the source of the proline accumulated? Is it transported from other parts of the plant or synthesized by the anther itself? What is the correlation between the accumulation process and anther development? In an attempt to answer some of these questions an early study was carried out with anthers where it was shown that on intact *Petunia hybrida*, the pollen contained free proline at levels as high as 2.6% of the dry weight (Linskens & Schrauwen, 1969). This *in vivo* study has now been complimented by an

investigation of anthers on detached branches of *Petunia*. The results of this investigation are given and discussed in chapter 2.

As to the function of the high content of free proline in pollen, one can first think of the metabolic fates of this amino acid. Britikov and his coworkers (1965) incubated pollen in an artificial medium containing [^{14}C]proline and found an evolution of $^{14}CO_2$. This may indicate that proline serves as a substrate for respiration, although from the amount of CO_2 released these authors concluded that the proportion of $^{14}CO_2$ derived from the carboxyl group was not very large. It should be pointed out however, that these results were obtained in long-term experiments (24 h) in the presence of a high concentration of sucrose which might interfere with proline metabolism. This problem is adressed in chapter 3 where a new medium containing no metabolizable substances is discribed. This medium proved to be superior to the conventional sucrose media for the germination of *Petunia hybrida* pollen. Using this new medium the degradation of [^{14}C]proline, labeled at different positions, was followed during germination and early tube growth of the pollen.

The role of proline in protein synthesis in pollen of *Petunia* is investigated in chapter 4. As undoubtedly, proline serves as a precursor for synthesis of proteins (Tupý, 1964; Britikov et al., 1965; Dashek & Harwood, 1974; Dashek & Mills, 1981), the present study concentrates on the question of whether the large proline pool is used, and to what extent in biosynthetic processes.

Besides the tremendous accumulation in pollen grains of many species, proline also accumulates in many plants under various stress conditions (Barnett & Naylor, 1966; Singh et al., 1972; Godzik & Linskens, 1974; Stewart & Lee, 1974; Chu et al., 1974; Treichel, 1975; Blum & Ebercon, 1976; Chu et al., 1978). Recently, Paleg and his coworkers (1981) reported that a number of solutes, including proline, protected enzymes from inactivation by heat. The above observations suggest that the abundant proline in pollen may have a protective function. Chapter 5 tackles the possible role of proline as a non-metabolic survival factor in pollen. Pollen of *Lilium longiflorum* was selected in this study because its natural free proline content is low and the endogenous proline concentration can be manipulated by changing the composition of the incubation medium. A high concentration of proline was found to protect pollen germination from

unfavorable temperatures. The protective effect on respiration and protein synthesis in pollen after heat shock is discussed in this chapter.

PROLINE ACCUMULATION DURING ANTHER DEVELOPMENT IN *PETUNIA HYBRIDA*

ABSTRACT

The accumulation of proline in anthers of *Petunia* was followed during flower bud development. Accumulation proceeded continuously from the microspore stage to anthesis except for an intervening period when bud length was between 15 and 35 mm. The second rise in proline content coincided with the stage of anther desiccation. The metabolism of proline was studied in isolated branches fed with [^{3}H]proline and [^{14}C]glutamine. Three factors were found to be involved in building up the proline content of the anthers: I. transport of proline from the leaves; II. transport of precursors, e.g. glutamate, and conversion to proline; III. high stability of proline in the anthers.

1. INTRODUCTION

In the pollen of many species the content of free proline is very high (Bathurst, 1954; Virtanen & Kari, 1955; Britikov & Musatova, 1964). Proline has been assumed to play an important role in pollen germination, tube growth and successful fertilization (Tupý, 1963; Dashek et al., 1971; Britikov et al., 1965, 1970; Pálfi et al., 1981; Zhang et al., 1982; Zhang & Croes, 1983 a). The kinetics and the mechanism of proline accumulation are unknown. The simplest mechanism involves transport from other parts of the plant of proline which is trapped in the developing anther.

Alternatively, proline might be synthesized in the anther itself from imported precursor molecules. Although it seems likely that the leaves are the source of proline or its precursors, there is no proof on this point. Neither is it known at what stage of anther development proline is accumulated.

An situation analogous to proline accumulation in the anther was found in leaves of water-stressed plants. The conversion of proline to glutamic acid was reported to be inhibited under water-stress (Aspinall et al., 1974) as was proline oxidation in barley leaves and in isolated maize mitochondria (Stewart et al., 1977; Sells & Koeppe, 1981). Also in stressed barley leaves, fixation of carbon dioxide by concurrent photosynthesis was found to be required for proline accumulation (Aiyar et al., 1980). Not surprisingly, accumulation of proline in barlay was found to correlate with abscisic acid level (Stewart, 1980).

Petunia hybrida is one of the species characterized by a large amount of proline in pollen (Linskens & Schrauwen, 1969; Zhang et al., 1982). In our study on proline accumulation in this species we concentrated on aspects of transport of proline and its precursor and on proline-glutamate interconversions in isolated branches bearing one developing bud.

2. MATERIALS AND METHODS

2.1. Plant material

Plants of *Petunia hybrida*, clone W166H, were grown in a greenhouse with artificial light at 15,000 lx and a photoperiod of 18 h. Only vigorous young plants were used for the experiments.

2.2. Assay of amino acids in anthers

For each determination at least 5 - 10 buds of the same length of the corolla were collected from the plants and all the anthers were gathered. The pool of free amino acids was extracted from the anthers by 70% ethanol containing 3 $mmol \cdot l^{-1}$ citric acid. The extract was shaken with 2 volumes of chloroform. After centrifugation or standing at 0 - 4°C overnight the

water phase was collected, dried and kept at -20°C until analysis. All the samples were assayed with an amino acid analyzer (JEOL, JLC-6AH, Tokyo, Japan) on a 6x600 mm column packed with LCR resin. To determine the ratio of dry to fresh weight, anthers from at least 20 buds were used for each determination. The anthers were weighed before and after drying in an oven at 90°C for 24 h.

2.3. Culture of branches

Branches of 20 - 25 cm were cut from the plants and trimmed. Of each branch only one bud, the corolla of which was about 10 mm, remained with 2 adjacent pairs of leaves. Five cm of the lower end of the stem were removed under water and the branch was transfered into a test tube (100x28 mm) filled with culture medium without isotopes. The medium was a modification of Hoagland's (Arnon & Hoagland, 1940) medium. In all the labeling experiments, a branch was given 1.4 MBq of [^{3}H]proline and 15 KBg of [^{14}C]glutamine in 50 µl of the culture medium at the cut end placed in a tiny tube. Just before the last droplet was taken up, another 100 µl of medium without isotopes was given to the branch. The feeding with unlabeled medium was repeated twice to make sure that all the isotope was taken up. The branches were then immediately put in the culture medium in the large test tubes. Great care was taken to assure that no air could enter the stem. All the branches were incubated up to 5 days in the test tubes in a large glass chamber in a hood under similar conditions as in greenhouse.

2.4. Sample fractionation

At 1-day intervals, 8 - 10 branches were gathered and used for amino acid and radioactivity determinations. The branches were separated into 6 parts: anthers, pistils, corolla, sepals, leaves and flower stalks. All samples except the anthers were cut into small pieces with a razor blade. After fresh weight determination, the samples were extracted with 3 mmol·l^{-1} citric acid in 70% ethanol. Because of the size of the leaves and the sepals, only parts of them were used for extraction.

2.5. Determination of radioactivity in proline and glutamate

The 70% ethanol extracts of the 6 fractions were shaken with chloroform as described above. The water phase was applied to an anion exchange resin (Dowex 50WX8). The amino acids adsorbed by the resin were eluted by 5 $mol \cdot l^{-1}$ ammonia. The ammonia was removed from the eluate by drying under a stream of N_2. The sample was redissolved in water and cochromatographed with 12.5 nmol of both proline and glutamic acid on 10x10 cm cellulose TLC plates (cut from cellulose precoated plate, 0.5 mm in thickness, 20x20 cm, PLC, MERCK, Darmstadt, West Germany). Up to 10 small plates were developed simultanously in a special glass rack. Amino acids are separated just as well as on large plates in a much shorter time. The plates were developed in iso-propanol-methylethylketone-HCl, 1 $mol \cdot l^{-1}$, (12:3:5, v/v/v) in the first dimension and phenol-H_2O-3% NH_3 (3:1:12, w/v/v/) in the second. Spots of proline and glutamic acid were visualized with ninhydrin reagent and scraped off into acified water (pH 3). The samples were allowed to stand overnight, taken up in 4 ml of Aqualyte (Baker, Deventer, Netherlands) and counted in a liquid scintillation analyzer (PHILIPS PW-4540, Eindhoven, Netherlands).

2.6. Radiochemicals

L-[2,3,4,5-^{3}H]proline, 4.03 $TBq \cdot mmol^{-1}$, and L-[U-^{14}C]glutamine, 1.48 $GBq \cdot mmol^{-1}$ were purchased from the Radiochemical Centre, Amersham, UK.

3. RESULTS

3.1. Development of anthers and accumulation of proline

The length of the flower buds used in our experiments was in the range of 5 to 55 mm, which corresponds to the stages of microspore to mature pollen grain one day before anthesis. This range is traversed by the developing flower bud in 7 - 8 days. During early development both fresh and dry weight of the anthers increased (Fig. 2-1). After the 35 mm bud stage the fresh weight of the anthers dropped quickly but the dry weight remained

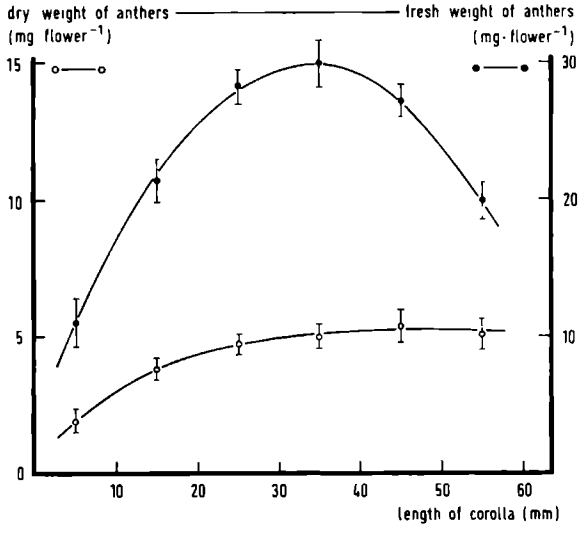

Figure 2-1. Dry weight (—○—○—) and fresh weight (—●—●—) of anthers of *Petunia* during flower bud development. Determination started at the microspore stage and ended one day before anthesis. For each determination, the anthers of 20 flower buds were pooled. Vertical bars indicate standard error (n = 3).

almost constant. In this late period of pollen development water content in the anthers decreased by 40%.

Free proline accumulates in the anthers from a very early stage of development (Fig. 2-2). The rate of accumulation is very high and proline content increases by a factor 3 when the flower buds grow from 5 to 15 mm. The accumulation is interrupted at a bud length between 15 and 25 mm. After this lag accumulation is resumed at almost the same rate as before and leads to a free proline pool in the anthers of up to 1.8% dry weight by the last day before anthesis. For sake of comparison, the change in glutamate, which is usually a predominant component in the amino acid pool, was followed. Glutamate content increases slowly at the early stage of development and then decreases. At the end of the development the content of it was about 0.37% of the dry weight.

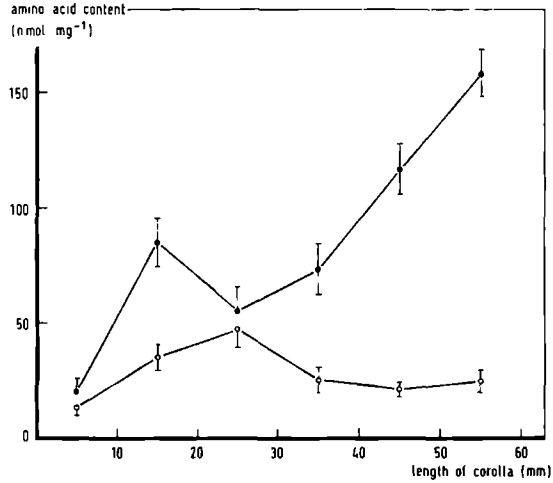

Figure 2-2. Amount of proline (—●—●—) and glutamate (—○—○—) in developing anthers of *Petunia*. The anthers of 5-10 buds were used for each determination.
Vertical bars indicate standard error (n = 5).

The composition of the free amino acids pools in the pistil, corolla, sepals, flower stalk and the 4 leaves adjacent to the flower bud was also followed during development. Free proline content in these parts of the plants remains very low throughout and the total pool of all amino acids is also smaller than in anthers (data not shown). A control experiment showed no substantial differences in the development of anthers and in amino acid content in anthers or the other parts of the plant between the isolated branches cultured for 5 days and the plants grown in the greenhouse, except a slight increase in proline content (maximally not more than 1 nmol·mg^{-1} during the incubation) in the leaves of the isolated branches.

3.2. Mechanism of proline accumulation

The accumulation of proline in the anther (Fig. 2-2) poses several ques-

tions concerning the metabolism of the amino acid in the anther. One of them is whether proline is transported as such from other parts of the plant or is synthesized in the anther from imported precursor molecules. If the latter possibility is true, glutamate could be the precursor or at least an intermediate. Another question concerns the rate of proline degradation. Although the amino acid is very reactive, an extensive conversion would not be expected in the anther when proline accumulates in such large amounts. To get some insight in these problems of proline metabolism, we labeled branches simultaneously with [^{3}H]proline and [^{14}C]glutamine.

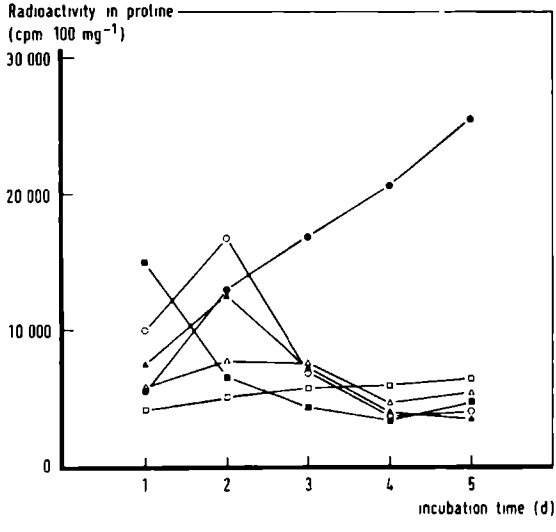

Figure 2-3. Distribution of [^{3}H]proline in different parts of isolated branches of *Petunia* fed with [^{3}H]proline, 1.4 MBq·branch^{-1}, at zero time. (—●—●—) anthers; (—○—○—) pistil; (—▲—▲—) corolla; (—△—△—) sepals; (—■—■—) leaves and (—□—□—) flower stalk.
The proline count was measured on ethanol extracts separated by 2-dimensional thin-layer chromatography.

The distribution of label in proline and glutamate was followed for 5 days. At one-day intervals, the amino acids were extracted from the different parts of the branches and separated by thin layer chromatography.

3.3. Distribution of labeled proline in the cultured branches

During the incubation of 5 days, the content of [^{3}H]proline in the leaves decreases continuously and at the same time it slowly increases in the flower stalk. In the pistils, corolla and sepals, it quickly decreases after the second day of incubation. Anthers were the only part of the branches in which [^{3}H]proline accumulates throughout the labeling period. By the end of incubation the content of [^{3}H]proline in the anthers is at least 4 times the amount found in the other parts (Fig 2-3). A calculation of [^{3}H]proline distribution shows that on the first day 99.2% of the total [^{3}H]proline is present in the leaves and only 0.1 % is in the anthers. After 5 days [^{3}H]proline in leaves decreases to 81.9% but in contrast it continuously increases in anthers up to 9.4%. Only 7.1 % of total [^{3}H]proline was recovered from pistil, corolla and sepals although the total weight of these parts of the flower bud is 3.8 times that of the anthers.

3.4. Conversion of glutamine to proline

In the same experiment, the ratio of ^{14}C in proline over that in glutamate was used as the indicator of the extent of proline synthesis from glutamate (Fig. 2-4). During the 5 days of incubation, the ratio in anthers is very high in a range of 55 - 70%. A ratio lower than 25%, in general, is found in pistil, corolla, sepals and the flower stalk. Apart from the 4th day, the ratio in leaves is lower than in anthers but higher than in the other parts of the branch (Fig. 2-4). This result would indicate that in the anthers proline is synthesized throughout the period of investigation.

3.5. Degradation of proline

The ^{3}H count in glutamate over the count in proline is indicative of the extent of degradation of proline in the branch, as the proline carbon skeleton enters the citrate cycle via glutamate and then is either degraded

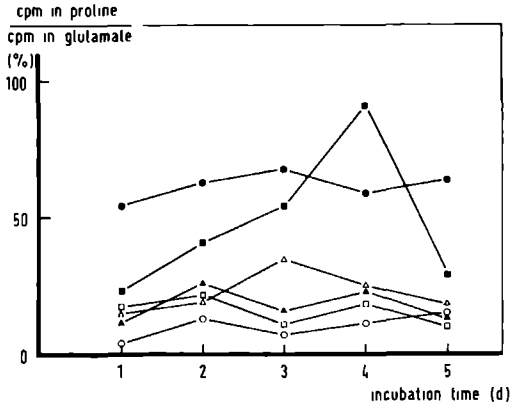

Figure 2-4. Ratio of [^{14}C]proline to [^{14}C]glutamate in different parts of isolated branches of *Petunia* fed with [^{14}C]glutamine, 15 KBq· branch^{-1}. Other details as in Fig. 2-3.

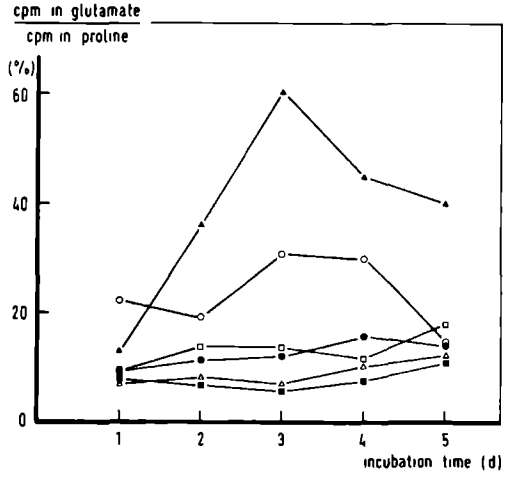

Figure 2-5. Ratio of [^{3}H]glutamate to [^{3}H]proline in different parts of isolated branches of *Petunia* fed with [^{3}H]proline, 1.4 MBq·branch^{-1}. Other details as in Fig 2-3.

to CO_2 or converted into other compounds. The ratio is shown in Fig. 2-5. It is low in the leaves and the sepals and somewhat higher in the anthers and in the flower stalk. This suggests that in these 4 organs proline degradation is not extensive. In contrast, much higher ratios are found in the pistil and the corolla.

4. DISCUSSION

The experiments on the mechanism of proline accumulation were started with 10 mm buds which are actively accumulating both proline and glutamate at least during the first part of the incubation period (Fig. 2-2). There is little doubt that the leaves serve as proline source and the anthers as proline sink (Fig. 2-3). However, it might be that proline label is transferred to the anthers as part of a compound different from proline. Such a process would involve proline conversion in the leaves and reconversion in the anthers. The very low [^{3}H]glutamate to [^{3}H]proline ratio in leaves of [^{3}H]proline labeled branches (Fig. 2-5), which is indicative of an insignificant proline to glutamate conversion, argues against this possibility. Approximately the same ratio is found in the flower stalk and this is a second argument for transport of proline in undegraded form. In contrast, the ratio is high in pistil and corolla. This would be expected as these organs develop rapidly during the period of observation in the absence of photosynthesis. Proline could be degraded for providing energy and building blocks for synthetic purposes.

The concept of proline accumulation as a result of proline transport from the leaves implies a high stability of proline in the anthers. This idea is supported by the low ratio of [^{3}H]glutamate to [^{3}H]proline in anthers (Fig. 2-5) and by numerous reports on proline accumulation in water-stressed leaves (Barnett and Naylor 1966; Aspinall et al., 1974; Singh et al., 1972; Stewart et al., 1977; Sells and Koeppe, 1981). In fact, the dramatic loss of water during the second part of pollen maturation (Fig. 1-1) would suggest that the increase in proline content during this period (Fig. 2-2) and the analogous process in water-stressed leaves are based, at least partially, on the same mechanisms. On basis of this concept of proline accumulation in anthers the extent of proline transport

can be estimated from the proline content in the anthers and the specific activity in the leaves. The estimate obtained in this way is 19 $nmol \cdot mg^{-1}$ and accounts for about 40% of proline accumulated during the 5 days of observation (Fig. 2-2). To what extent does glutamate serve as a precursor or intermediate of proline synthesis in the anthers? The decline in glutamate content and the concomitant rise in proline content during the second period of anther maturation are certainly not at variance with the notion of a conversion of glutamate to proline in the anther (Fig. 2-2). Fig. 2-4 shows that in the flower stalk as in most parts of the flower bud, the ratio of [^{14}C]proline to [^{14}C]glutamate in [^{14}C]glutamine-labeled branches is 15% which is lower than in the leaves during the whole incubation. This indicates that the flow of [^{14}C]glutamate toward the flower bud exceeds that of [^{14}C]proline. A ratio of 60% is found in the anther which makes it very likely that glutamate to proline convertion takes place in the anther and contributes to proline accumulation. However, it is very difficult to quantify this contribution as glutamate enters in many other metabolic pathways. In conclusion, three factors have been found to lead to proline accumulation in anthers: transport of proline from the leaves, synthesis of proline in the anthers from or *via* glutamate and finally a low degradation rate of the proline which is accumulated.

CHAPTER 3

PROLINE DEGRADATION IN POLLEN

PART (1)

A NEW MEDIUM FOR MEASURING RESPIRATION IN THE ABSENCE OF SUCROSE

ABSTRACT

Poly-ethylene glycol (PEG)-400 was found to be superior to sucrose as the major component in media for the germination of *Petunia hybrida* pollen. PEG promotes pollen tube growth considerably more than sucrose. Pollen tubes grown on PEG resemble the *in vivo* tubes much more than those grown on sucrose. The respiration rate of pollen germinating on sucrose is 33% higher than on PEG; the extra energy produced is clearly not related to tube growth.

1. INTRODUCTION

As pollen germination *in vitro* is a routine method in several research fields, many incubation media have been established during the last decades (Stanley & Linskens, 1974). In most media a soluble sugar, generally sucrose, is used both as an osmoticum and as a substrate for respiration. Because high concentrations of metabolizable sugars might interfere with

the utilization of other substrates, several substances such as mannitol, sorbitol, penta-erythritol, ethylene glycol, propylene glycol and poly-propylene glycol, which are not or not easily metabolized in pollen, have been proposed as substitutes for sucrose. These compounds were tested on lily (Dickinson, 1968, 1978) and *Petunia* (Stanley & Linskens, 1964) pollen. It was found that on none of them did pollen tubes grow better than on sucrose.

Poly-ethylene glycol (PEG), wich is also not metabolized in plants and can provide water potentials over a wide range (Stenter et al., 1981), has been tested by Dickinson (1968) on lily pollen with unsatisfactory results. In this report we propose a simple PEG-400 medium for germinating *Petunia* pollen which yields about the same germination percentage but promotes tube growth considerably more than the conventional sucrose medium.

2. MATERIAL AND METHODS

2.1. Plant material

Plants of *Petunia hybrida*, clone W166H (incompatibility alleles S_2S_3) and clone W43 (incompatibility alleles S_1S_1) were grown in the greenhouse with artificial light (15,000 lx) at a photoperiod of 18 h.

2.2. Pollination and germination

Pollen of clone W166H was harvested on the day of anthesis, mixed thoroughly and pollinated to the surface of mature stigmas of clone W43. Four hours after pollination the pistils were collected and fixed in a mixture of formalin, acetic acid and ethanol, 70% (1:1:18 v/v/v). Stigmas with attached styles were softened in 8 $mol \cdot l^{-1}$ NaOH, squashed and stained in aceto-carmine. The diameters of pollen tubes growing in the pistil were determined microscopically.

Pollen from the same clone was equilibrated for 2 h in air at a relative humidity of 100% and germinated *in vitro* at 25°C for 3 h with continuous shaking in solutions containing sucrose or PEG-400 (British Drug Houses) at different water potentials. The sucrose solutions yielding the

water potentials desired (ψ = -2.5 to -40 bar) ranged from 0.1 to 1.1 $mol \cdot l^{-1}$. PEG concentrations were between 0.08 and 0.65 $mol \cdot l^{-1}$ for the same water potential range. Boric acid, 100 $\mu g \cdot ml^{-1}$, was included in all media. The concentration of pollen was at 5 mg (fresh weight) per ml. After culturing, the pollen was stained with cotton blue (Datta & Naug, 1967) and the germination percentage was determined microscopically. From each medium 5 samples were counted. Pollen grains with tubes longer than half the diameter of the grain were considered to be germinated. Length and diameter of pollen tubes were determined under a projection microscope; at least 100 randomly selected tubes were counted in each determination.

2.3. Determination of water potential

All the media were carefully adjusted to selected water potentials determined by an osmometer. At the end of the incubation 1 ml of the culture was filtered and the water potential of the filtrate was determined.

2.4. Respiration

10 mg of pollen from clone W166H were germinated in 2 ml of sucrose or PEG-400 medium at 25°C in a Warburg flask and the respiration rate was measured manometrically (Umbreit et al., 1964). Three pollen samples per treatment were assayed in each experiment.

3. RESULTS

3.1. Pollen germination and tube growth

Pollen of *Petunia hybrida*, clone W166H, incubated at different water potentials in sucrose or PEG-400 medium started germination about 0.5 h after the onset of incubation. After 3 h germination percentages ranged from 29 to 61% (Fig. 3-1-1-b). Both curves show a broad optimum at about -10 bar (0.36 $mol \cdot l^{-1}$ for sucrose, 0.29 $mol \cdot l^{-1}$ for PEG). With sucrose the germination percentage is somewhat higher over virtually the whole range;

at the optimum the difference is approximately 5%.

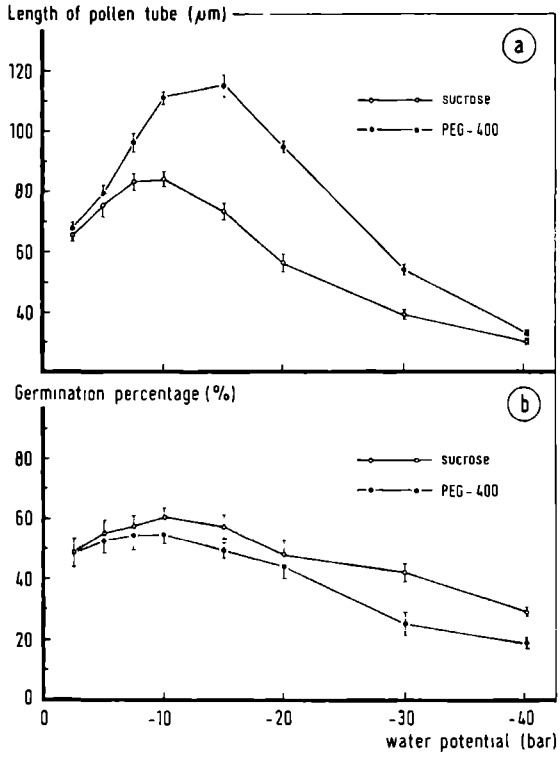

Figure 3-1-1. Germination and tube growth of *Petunia pollen* in sucrose and PEG-400: Pollen of *Petunia hybrida*, clone W166H, was germinated at 25°C for 3 h. The data are means of 5 determinations. At least 100 pollen tubes were randomly selected for tube length measurement (a). For determining the germination percentage at least 200 pollen grains were screened (b). Vertical bars indicate standard deviations.

Much more difference is observed in the length of the pollen tubes (Fig. 3-1-1-a). In PEG-400 medium the optimal water potential is -15 bar,

5 bar lower than in sucrose medium. It is remarkable that pollen tubes are longer in PEG medium and that tube length varies over a much wider range than germination percentage.

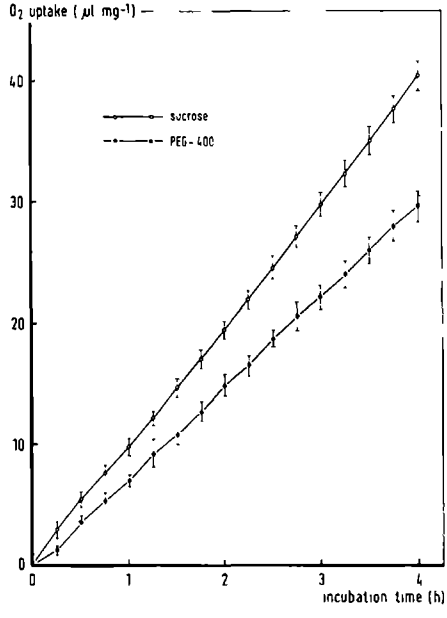

Figure 3-1-2. Oxygen consumption: Pollen of *Petunia hybrida*, clone W166H, was incubated at 25°C in PEG-400 (ψ = -15 bar) or sucrose medium (ψ = -10 bar) and assayed by Warburg manometry. The data are means of 3 determinations.
Vertical bars indicate standard deviations (n = 3).

Morphological differences between pollen tubes grown on sucrose and on PEG at water potentials optimal for elongation are striking. In general, the PEG-grown pollen tubes resemble more closely tubes growing in the style than those grown on sucrose (Fig. 3-1-3). On PEG-400 the tubes grow

faster and more uniformly and their appearance is less crooked than in sucrose medium. Moreover, the diameter of the tubes in PEG-medium (8.8 μm) is nearly as *in vivo* (8.2 μm), but greatly differs from the diameter of the tubes in sucrose medium (12.2 μm).

3.2. Changes in water potential

After 3 h of incubation the water potential has dropped from -10 bar to -11.5 bar in the sucrose solution and from -10 to -10.6 bar in PEG. This might indicate that with sucrose more material is released into the medium than with PEG-400.

3.3. Respiration

Respiration of pollen incubated at water potentials optimal for tube growth are shown in Fig. 3-1-2. In both media the uptake of O_2 is almost linear with time. The rates of oxygen consumption as calculated from the regression lines are 10.0 $\mu l \cdot mg^{-1} \cdot h^{-1}$ in sucrose and 7.5 $\mu l \cdot mg^{-1} \cdot h^{-1}$ in PEG-400 medium. Thus possibly the sucrose is used as an exogeneous substrate for respiration. Since the growth of the pollen tubes on PEG is faster, it is clear that the additional oxygen consumption in the presence of sucrose is not related to tube growth.

4. DISCUSSION

The initiation of the *Petunia* pollen tube *in vitro* is apparently affected by the water potential of the medium (Fig. 3-1-1-b). The similarity of the two germination curves indicates that sucrose primarily acts as an osmoticum. There is, however, some difference in germination percentage between the two kinds of media as the percentage is 5% higher with sucrose under optimal conditions. Before and during tube initiation ATP will be needed for protein synthesis, synthesis of wall material and other energy consuming processes. Thus sucrose might also serve as an energy source for germination. This view is supported by the observation that sucrose promotes respiration by 33% over the incubation without sucrose (Fig. 3-1-2). In

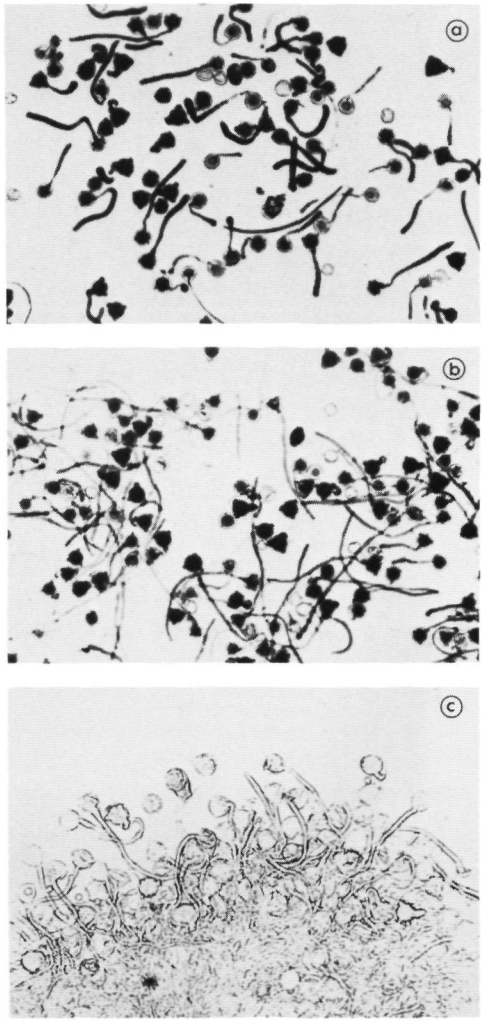

Figure 3-1-3. Microphotographs of *Petunia* pollen germinated (a): in sucrose medium, (b): in PEG-400 medium and (c): on the pistil. Magnification: 125 x.

addition to a role of sucrose in germination the sugar might be involved in some way in the increase in volume of the pollen tubes which is considerably greater on sucrose than on PEG.

At all water potentials, the average length of the pollen tubes is greater on PEG than on sucrose (Fig. 3-1-1). This could mean that concentrated sucrose is harmful for the tube growth. If this is true, the water potential optimal for elongation would be expected to be less negative with sucrose than with PEG. This is indeed what is found (Fig. 3-1-1-a). A harmful effect of sucrose might also be inferred from the changes in the water potential of the incubation medium. In the case of sucrose the water potential decreases 2.5 times more than in PEG during incubation, at least partly because more material leaks from the pollen into the medium. For instance, the release of amino acids into the sucrose medium is 32% larger than into PEG medium (data not shown). This might indicate that the permeability of the membrane is altered by the high sucrose concentration. Another factor leading to the decrease in water potential observed might be the hydrolysis of some sucrose by an extracellular invertase (Tupý, 1960; Dickinson, 1967).

In the PEG medium there is no exogenous substrate for respiration. The endogenous substrate may be carbohydrate but also proline could be involved (Britikov et al., 1965; Britikov & Linskens, 1970). The endogenous substrate supports ATP synthesis sufficient for germination and for tube growth during at least the first hours.

As *Petunia* pollen grains normally germinate and even produce much longer pollen tubes on PEG-400, this compound is more satisfactory than sucrose as the major component of the incubation medium for *in vitro* studies.

PART (2)

DEGRADATION OF PROLINE DURING GERMINATION AND EARLY TUBE GROWTH

ABSTRACT

Proline degradation in *Petunia* pollen germinated *in vitro* was studied in cultures supplemented with [^{14}C]proline labeled at different positions. Despite its abundance in the cell, this amino acid is only a minor substrate for respiration. Proline is partially converted via the citrate cycle into metabolites which can be traced in the ethanol-insoluble fraction of the cellular constituents. The fact that this conversion is much more extensive than proline use in respiration suggests that, in germination pollen, the citrate cycle mainly serves for synthetic purposes. There are no indications that proline carbon is used for feeding of other amino acid pools used for protein synthesis.

1. INTRODUCTION

A number of studies have been undertaken to elucidate the role of proline which accumulates in large quantities in the pollen of many plant species. In *Petunia* pollen, proline comprises 2.6% of the dry weight (Linskens & Schrauwen, 1969). Proline may act as a relatively harmless solute protecting pollen from unfavorable temperatures by reducing the water potential inside the grain (Zhang & Croes, 1983 a). Part of the proline pool is used for protein synthesis during germination and outgrowth of the tube (Britikov et al., 1965; Zhang et al., 1982). In other tissues the amino

acid has been found to serve as a nitrogen source for a number of amino acids and other nitrogenous compounds (Britikov et al, 1970). In leaves, proline is degraded via glutamate and its carbon moiety is recovered in several amino acids and ninhydrin-negative compounds (Stewart et al., 1977). In pollen germinated *in vitro*, feeding with [U-^{14}C]proline leads to the formation of labeled aspartate, glutamate and glutamine (Britikov et al., 1965). From the amount of CO_2 released these authors concluded that the proportion of $^{14}CO_2$ derived from the carboxyl group was not very large. However, the results were obtained in long-term experiments (24 h) in the presence of a high concentration of sucrose which might interfere with proline metabolism. The presence of sucrose can be avoided by application of a germination medium containing no metabolizable carbon source (Zhang & Croes, 1982). Short-term labeling experiments, however, are seriously hampered by isotope dilution due to mixing with the large internal proline pool and with the proline excreted into the medium upon germination (Linskens & Schrauwen, 1969; Zhang et al., 1982). Nevertheless, an attempt was made to follow proline degradation in pollen during germination and early tube growth by the use of [^{14}C]proline labeled at different positions.

2. MATERIAL AND METHODS

2.1. Experimental procedures

All materials and techniques used have been described previously (Zhang & Croes, 1982; Zhang et al., 1982) except for the following.

Pollen was equilibrated in water-saturated air for 2 h and then germinated *in vitro* in a medium consisting of polyethylene glycol-400 (0.3 mol·l^{-1}) and boric acid (1.6 mmol·l^{-1}).

Total CO_2 production was followed manometrically (Umbreit et al., 1964) at 25°C. Radioactive CO_2 formed by degradation of [^{14}C] labeled substrates was trapped in KOH (1.8 mol·l^{-1}) and counted after mixing with Aqualyte (Baker). In these experiments, L-[^{14}C]labeled amino acids were added to the medium 1 min after the onset of incubation at 18.5 kBq·ml^{-1}, DL-amino acids at twice that activity.

2.2. Radiochemicals

DL-[1-^{14}C]proline, 1.85 GBq·mmol^{-1}, DL-[5-^{14}C]proline, 1.52 GBq·mmol^{-1}, L-[1-^{14}C]glutamic acid, 2.07 GBq·mmol^{-1} (Commissariat à l'Energie atomique, France) L-[U-^{14}C]proline, 8.88 GBq·mmol^{-1}, (ICN Pharmaceuticals, USA) and L-[U-^{14}C]glutamic acid, 10.55 GBq·mmol^{-1} (Radiochemical Centre, Amersham, UK) were used.

3. RESULTS

3.1. Uptake of proline in pollen

Upon incubation of *Petunia* pollen under standard conditions an amount of proline of 90 nmol per mg pollen is released which leads to a proline concentration of 0.45 mmol·l^{-1} in the germination medium. Part of the proline is taken up again in a relatively slow process (Table 3-2-1). Only after 2.5 h is the pool saturated with isotope.

Table 3-2-1. Uptake of proline by germinating *Petunia* pollen. Pollen was incubated at 5 mg·ml^{-1} in germination medium supplemented with L-[U-^{14}C]proline, 37 kBq·ml^{-1}

Time (h)	Proline uptake (cpm·mg^{-1})	Time (h)	Proline uptake (cpm·mg^{-1})
0.5	30,631	2	68,871
1	60,855	2.5	73,770
1.5	69,464	3	73,934

3.2. Proline as respiration substrate

Since the germination medium does not contain any energy supply at the

onset of the experiment, respiration is dependent on endogenous reserves, part of which may be excreted during early germination and reabsorbed later on. To estimate the role of proline in respiration, we labeled the germination medium with [U-^{14}C]proline and measured total CO_2 production and evolution of $^{14}CO_2$ for 3 h (Fig. 3-2-1). The rate of CO_2 production is fastest at the beginning and slows down to a new constant level after 1 h. The rate of $^{14}CO_2$ release from proline continuously increases as might be expected from the slow uptake kinetics. At the end of the incubation period the contribution of proline to the total CO_2 production as measured in this way amounts to 0.7%.

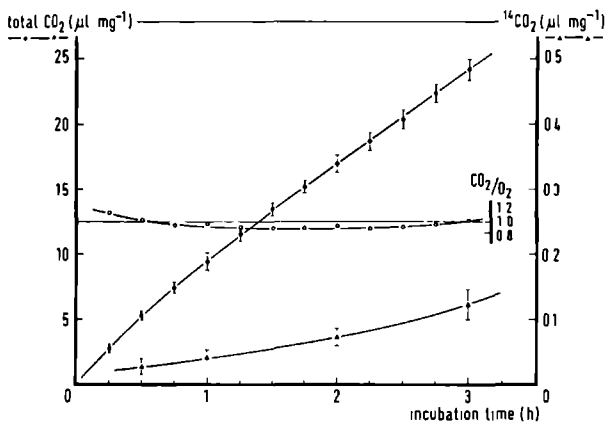

Figure 3-2-1. Total CO_2 production (—●—●—), CO_2 evolution from proline (—▲—▲—), and respiratory quotient (—○—○—) of *Petunia* pollen germinated *in vitro*. Pollen was incubated at 5 mg·ml^{-1} in a germination medium containing 18.5 kBq·ml^{-1} of L-[U-^{14}C]proline. The specific activity of proline in the medium was estimated on basis of its concentration which results from proline excretion by the pollen. For the calculation of CO_2 evolution from proline, immediate and complete mixing with the internal pool was assumed. The respiratory quotient was calculated from O_2 absorption and CO_2 release during the preceding 15-min interval.
Vertical bars indicate standard error (n = 3).

The respiration quotient was calculated from the rates of CO_2 evolution in 15-min intervals. The variation observed (Fig. 3-2-1) suggests that the nature of the substrate for respiration more or less changes with the stage of development.

3.3. Nature of proline degradation

During respiration the proline carbon skeleton might be either completely degraded to CO_2 or part of it might leave the citrate cycle in a different form. To discriminate between these possibilities, we supplemented duplicate cultures with DL-[1-^{14}C]proline and L-[U-^{14}C]proline respectively. DL proline was added at twice the activity as L-proline since preliminary experiments had shown that only half of the DL-proline counts are taken up ($52 \pm 4\%$, $n = 4$). If the proline molecule were completely degraded, the $^{14}CO_2$ production would be the same in both cultures. Fig. 3-2-2-a shows that much less counts are freed from [U-^{14}C]proline which indicates that a considerable portion of the proline carbon is trapped in compounds different from CO_2. Additional evidence in support of this conclusion comes from an analogous experiment with L-[1-^{14}C] and L-[U-^{14}C]glutamate, an intermediate in proline degradation (Stewart et al., 1977). Essentially the same results were obtained (Fig. 3-2-2-b).

When proline enters the citrate cycle via glutamate, the carboxylic group in position 1 is converted into CO_2. The carbon atom in position 5 is freed as CO_2 in 50% of the molecules in the first turn of the cycle by decarboxylation of oxalosuccinate. Another 25% comes free by subsequent decarboxylation of α-ketoglutarate. The evolution of $^{14}CO_2$ from [5-^{14}C]-proline would be, therefore, maximally 25% lower than that from [1-^{14}C]-proline unless radioactive intermediates are drained off from the cycle in the first turn. The extent of this drainage was investigated in duplicate cultures containing [1-^{14}C] and [5-^{14}C]proline respectively (Fig. 3-2-3). It is seen that the amount of $^{14}CO_2$ evolved from the [5-^{14}C]proline is much less than 75% of the amount freed from the [1-^{14}C]form. This would mean that a considerable portion of the proline carbon leaves the citrate cycle in the first turn in a form different from CO_2.

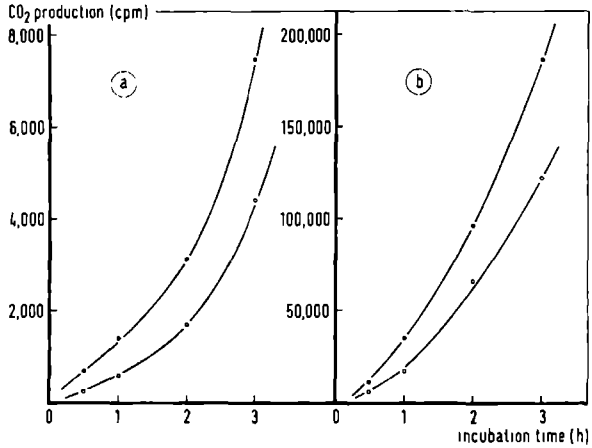

Figure 3-2-2. Release of $^{14}CO_2$ from differently labeled substrates by *Petunia* pollen germinated *in vitro*. Pollen was incubated at 5 mg·ml^{-1} in the presence of (a) [^{14}C]proline and (b) [^{14}C]-glutamate, 18.5 kBq·ml^{-1} except for [1-^{14}C]proline which was given in the DL form at 37 kBq·ml^{-1}. (—●—●—) [1-^{14}C] labeled substrate; (—○—○—) [U-^{14}C] labeled substrate.

3.4. Proline conversion and macromolucular synthesis

Direct evidence concerning the manner of proline conversion would come from a search for degradation products. However, 70% ethanol extracts from pollen cultures in the presence of [^{14}C]proline proved to contain proline as the only labeled compound after 2-dimensional thin layer chromatography. The absence of other detectable radioactive spots is without doubt due to the extensive dilution of label in the medium and inside the pollen grain.

The incorporation of proline carbon in the 70% ethanol insoluble fraction is shown in Table 3-2-2. The difference in incorporation of position 5 and position 1 carbon atoms indicates that one or more degradation products of proline serve as substrate for macromolecular synthesis. One possibility is that proteins are indirectly labeled via formation and incorporation of amino acids such as glutamate, alanine and aspartate.

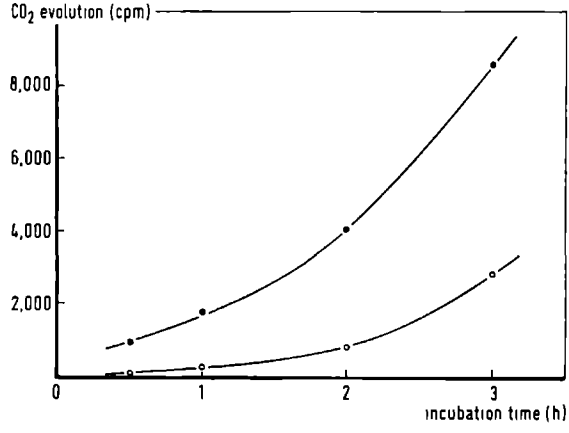

Figure 3-2-3. Evolution of $^{14}CO_2$ from DL-[^{14}C]proline labeled at different positions by germinating *Petunia* pollen. (—●—●—) [1-^{14}C] and (—○—○—) [5-^{14}C]proline.

To test this, we hydrolyzed the proteins from [5-^{14}C]proline labeled cultures of pollen and analyzed the hydrolysate by 2-dimensional thin layer chromatography. Only 2 labeled spots were found located at the proline and hydroxyproline positions.

Table 3-2-2. Incorporation of proline into 70% ethanol-insoluble cell material of *Petunia* pollen. Pollen was germinated in the presence of 1-position or 5-position labeled DL-[^{14}C]proline at an activity of 37 kBq·ml^{-1}. The analysis was performed after 3 h.

Carbon atom labeled	Proline uptake (cpm·mg^{-1})	Proline incorporation (cpm·mg^{-1})
1	77,022	847
5	83,957	2,273

The rate of total CO_2 production is fairly constant throughout the observation period except for a small decrease after the first hour. The steady increase in the rate of $^{14}CO_2$ evolution is, therefore, most likely due to the slow saturation of the internal pool with labeled proline (Table 3-2-1). Thus there is no evidence for the existence of a rapidly saturating proline pool for respiration. Such evidence has been obtained for proline incorporation into proteins where linear kinetics is found from the beginning on (Zhang et al., 1982). In the absence of similar indications for proline respiration, immediate and complete mixing of absorbed proline with the internal pool was assumed.

Even if CO_2 evolution from proline is calculated on basis of $^{14}CO_2$ production during the third hour, i.e. at saturation of the pool, and if the preferential degradation of the carboxyl group (Fig. 3-2-2) is taken into account, the maximal contribution of proline carbon to total respiration amounts to no more than 1 or 2%. Despite its abundance, proline is only a minor substrate for respiration.

The observation that 3-5 times more radioactivity evolves in CO_2 from [1-^{14}C]proline than from [5-^{14}C]proline leads to the conclusion that proline degradation is very incomplete. A rough calculation shows that 60-70% of the carbon atoms entering the citrate cycle presumably leaves in the form of one or more C_4 compounds. This would mean that the citrate cycle mainly serves for synthetic purposes. Our attempts to identify these compounds by thin-layer chromatography have failed. As a result of the tremendous dilution of label, radiocarbon can only be traced in the end products of metabolic pathways.

As could be expected from the incomplete proline degradation, the amount of label in the ethanol-insoluble material is much higher in pollen incubated with [5-^{14}C]proline than in [1-^{14}C]proline labeled pollen (Table 3-2-2). Unlike the situation in other species (Britikov et al., 1965), proline carbon is not measurably used for the synthesis of other amino acids since proline and hydroxyproline are the only labeled amino acid residues In protein hydrolysates from [^{14}C]proline fed pollen cultures. A reasonable alternative would be that via gluconeogenesis proline carbon becomes incorporated into carbohydrates which in turn would be used

for cell wall synthesis in the fast growing tip of the pollen tube.

CHAPTER 4

PROTEIN SYNTHESIS IN GERMINATING POLLEN OF *PETUNIA*: ROLE OF PROLINE

ABSTRACT

Pollen of *Petunia hybrida* was germinated in artificial medium. At the beginning of the incubation, a large amount of proline, which comprises about half of the total free amino acid pool, was released into the medium. Part of this proline is reutilized by the pollen. Uptake of radioactive amino acids and their incorporation into proteins were studied. The highest rate of protein synthesis was found directly after the onset of germination. The endogenous free proline pool was found to be compartmentalized; one of the compartments is the protein precursor pool; its size is probably much less than 50% of the total free proline in the pollen.

1. INTRODUCTION

Proline is one of the more abundant free amino acids in pollen. Almost without exception, the concentration of free proline in pollen is much higher than in pistil tissues (Britikov & Musatova, 1964). In pollen of *Petunia hybrida*, free proline comprises 2.6% of the dry weight (Linskens & Schrauwen, 1969). It is likely to play an important role in protein synthesis, especially in the synthesis of proline and hydroxyproline-rich proteins. Hydroxyproline-rich glycoproteins are prevalent in the cell wall (Lamport, 1977). As pollen tube elongation is accompanied by an extensive

production of cell wall material at the pollen tube tip, synthesis of these proteins might be of critical importance in tube development. The question arises, whether or not the large proline pool as a whole is available for protein synthesis. It might be that in the cell there are several pools with different functions in metabolism. Such a situation has been found many times especially in animal cells (Kipnis et al., 1961; Bidwell et al., 1964; Oaks, 1965; Holleman & Key, 1967; Van Venrooij et al., 1974; Airhart et al., 1974; Ilan & Singer, 1975).

In this report the uptake and incorporation of exogenously supplied proline are described and the involvement of the internal proline pool in protein synthesis is evaluated.

2. MATERIAL AND METHODS

2.1. Plant material

Pollen was collected on the day of anthesis from plants of *Petunia hybrida*, clone W166H, (incompatibility alleles S_2S_3) grown in a greenhouse under artificial light at 15,000 lx and a photoperiod of 18 h. After harvest, the pollen was stored overnight in a desiccator with silica gel at 0 - 4°C.

2.2. Germination of pollen

Before germination, the pollen was exposed to a relative humidity of 100% for 2 h. The pollen was subsequently germinated in a liquid medium consisting of 10% sucrose and 0.01% boric acid at 25°C, either in Erlenmeyer flasks with continuous shaking (130 strokes·min^{-1}) or in a mass culture vessel (Schrauwen & Linskens, 1967). The medium was sterilized by autoclaving. The concentration of pollen in the medium was at 5 mg (fresh weight) per ml. Germination percentage and pollen tube length were determined microscopically on at least 300 randomly selected pollen grains.

2.3. Determination of amino acid content

Samples containing 2.5 mg pollen were taken from the culture and washed 3 times in ice-cold culture medium. The pool of free amino acids in pollen was extracted 3 times with 3 ml 70% ethanol containing 0.1 mol·l^{-1} thiodiglycol and 3 mmol·l^{-1} citric acid. The combined extracts were shaken with 2 vol. chloroform, and the water phase was used for pool analysis. The residue was digested in 6 mol·l^{-1} HCl at 105°C for 24 h in a sealed tube under vacuum. The HCl was removed by evaporation before analysis. Free amino acids in the incubation medium were purified by adsorption to an anion exchange resin (Dowex 50WX8) prior to analysis. The samples were assayed with an amino acid analyzer (JEOL JLC-6AH) on a 6·600 mm columm of LCR resin.

2.4. Uptake and incorporation of radioisotopes

In most isotope experiments, 370 kBq·ml^{-1} [^{3}H]proline and 18.5 kBq·ml^{-1} [^{14}C]phenylalanine were added to the culture medium at various times, after the onset of incubation. After addition of label, samples were collected at 5-min intervals. Samples containing amino acids from the medium or the free pool were prepared as described above and mixed with Aqualuma (Baker). The counts in the residue remaining after ethanol extraction were considered to be in proteins. The material was solubilized in Lumasolve (Baker) and taken up in Lipoluma (Baker). The samples were counted in a liquid scintillation analyzer (Philips PW-4540).

2.5. Conversion of pool components

Amino acid pool fraction and protein hydrolysates were prepared from germinated pollen preincubated for 3 h with [^{14}C]proline or [^{14}C]phenylalanine, 18.5 kBq·ml^{-1}. The samples were subjected to 2-dimensional thin-layer chromatography on precoated cellulose plates (MERCK) with isopropanol-methylethylketone-1 mol·l^{-1} HCl (12:3:5, by vol.) and phenol-H_2O-3% NH_3 (3:1:12, w/v/v) as the solvents. Radioactive spots were detected by autoradiography on X-ray film (Du Pont Cronex 4), scraped off into scintillation fluid and counted.

2.6. Radiochemicals

L-[2,3,4,5-^{3}H]proline, 4 TBq·mmol^{-1} (Radiochemical Centre, Amersham, UK.), L-[U-^{14}C]proline, 8.9 GBq·mmol^{-1} (ICN Pharmaceuticals) and L-[U-^{14}C]-phenylalanine, 16.7 GBq·mmol^{-1} (ICN Pharmaceuticals) were used.

3. RESULTS

3.1. Pollen germination and tube growth

Pollen of *Petunia hybrida*, clone W166H, started germination after about 0.5 h and after 3 h incubation the germination percentage reached 65% and did not increase substantially thereafter (Fig. 4-1). Pollen tubes elongate almost linearly at a rate of 20 µm·h^{-1} throughout the experimental period.

3.2. Amino acid content in non-germinated pollen

The concentrations of 17 amino acids in non-germinated *Petunia* pollen are shown in Table 4-1. The most abundant amino acid in the free pool is proline. Its concentration amounts to 55% of the total amino acid pool and exceeds the proline in proteins by 70%. In contrast, of all amino acids phenylalanine is present at the lowest concentration in the pool. The ratio of free and bound phenylalanine is approximately 1:200. For this reason, phenylalanine was used for reference in the isotope experiments, since dilution of label by the internal pool would be virtually absent.

3.3. Changes in proline and phenylalanine content during germination

The amounts of proline and phenylalanine in germinating pollen are presented in Fig. 4-2. Within the first few minutes, the concentration of free proline in pollen drops sharply and then continuously decreases, but much slower. As a result, there is a fast initial increase in proline concentration in the medium. After 15 min of germination, the concentra-

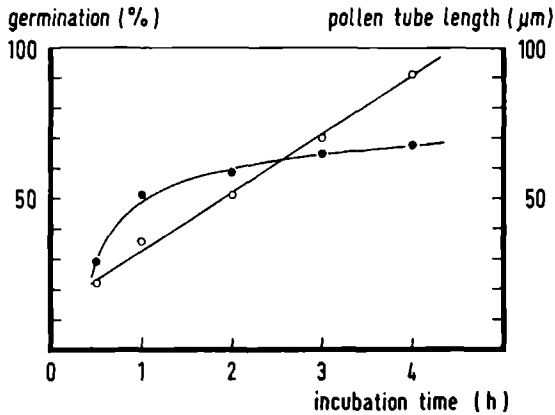

Figure 4-1. Germination and tube elongation of *Petunia* pollen in artificial medium. A sample containing at least 300 pollen grains was randomly taken and all the grains were counted microscopically. Only the pollen grain which had a pollen tube longer than half the diameter of the grain was considered as germinated. (—●—●—) germination percentage; (—o—o—) pollen tube length.

tion has risen to 0.8 $mmol \cdot l^{-1}$. As the total amount of proline in the medium plus pollen increases, some proline must be newly synthesized after germination. The content of phenylalanine in pollen is extremely low and amounts to no more than 0.8 $nmol \cdot mg^{-1}$ pollen. In isotope experiments, phenylalanine was added to the incubation medium at a final concentration of 0.75 $mmol \cdot l^{-1}$. As a result, the free phenylalanine pool increases to 4 - 8 $nmol \cdot mg^{-1}$ pollen within 25 min. This amount of phenylalanine greatly exceeds the size of the original pool. Throughout the experimental period, the ratio of bound proline and phenylalanine remains constant at 1.2. Therefore, it was assumed that proline and phenylalanine are incorporated at the same ratio.

Table 4-1. Amino acid composition of free pool and protein in non-germinated *Petunia* pollen. The data are means of 3 experiments

Amino acid	Free pool ($nmol \cdot mg^{-1}$) (1)	Hydrolysate ($nmol \cdot mg^{-1}$) (2)	(1)/(2) (%) (3)
Asp	24.3	267.2	9.1
Thr	30.2	121.3	24.9
Ser	13.2	166.7	7.9
Glu	43.3	255.9	16.9
Pro	213.9	125.1	170.1
Gly	2.9	218.5	1.3
Ala	34.1	213.0	16.0
Cys	0.5 [a]	10.3	4.9 [b]
Val	6.5	141.2	4.6
Met	1.4	19.1	7.3
Ileu	1.8	113.4	1.6
Leu	0.9	205.6	0.4
Tyr	0.5 [a]	70.3	0.7 [b]
Phe	0.5 [a]	95.2	0.5 [b]
Lys	3.0	168.3	1.8
His	4.8	49.8	9.6
Arg	7.8	98.7	7.9

[a] Free pool size of the amino acid is less than 0.5 $nmol \cdot mg^{-1}$

[b] The value was calculated on the assumption that the amount in the pool is 0.5 $nmol \cdot mg^{-1}$ or less.

3.4. Uptake and incorporation of amino acids

The uptake of [^{3}H]proline and [^{14}C]phenylalanine and their incorporation into proteins is shown in Fig. 4-3. Apart from the quantitative differences, the patterns of uptake and incorporation are similar at all four labeling periods. For both amino acids, uptake exceeds incorporation by

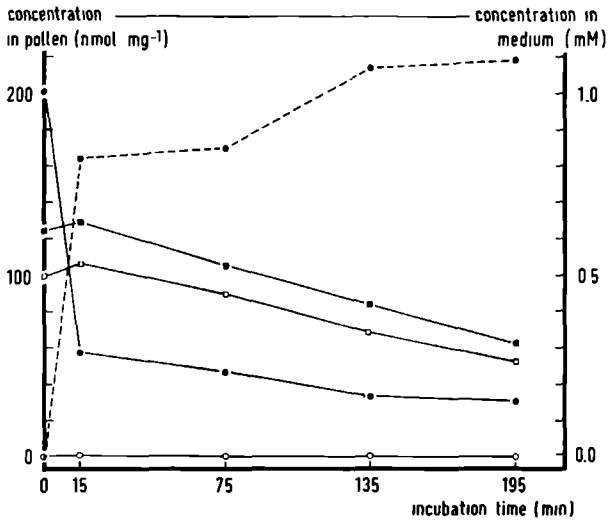

Figure 4-2. Proline and phenylalanine in *Petunia* pollen and incubation medium during germination *in vitro*. Data for proline and phenylalanine in the pool are means of 3 determinations, the other points in the graph are means of two experiments; (—●—●—) free proline in pollen; (—○—○—) free phenylalanine in pollen; (—■—■—) proline in protein; (—□—□—) phenylalanine in protein; (--●--●--) proline in medium.

a wide margin, so that the former process can not be limiting for the latter. The rate of uptake of both amino acids is linear with time during the first 25 min. The zero order of the kinetics of incorporation of both amino acids is the most striking feature. As it is reasonable to assume that the actual rate of protein synthesis does not dramatically change during the labeling period, the linear incorporation kinetics would indicate that the specific activity of the protein precursors become constant soon after addition of label. For phenylalanine this result is expected, as the internal pool is small compared to the amino acid taken up from the medium, and pool dilution would thus be negligible. For proline, however, the result is unexpected and can only be explained by an absent or very incomplete mixing of radioactive proline with the endogenous pool.

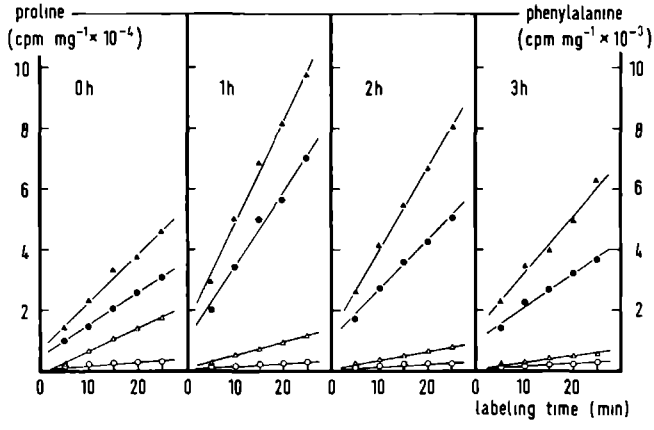

Figure 4-3. Uptake and incorporation of [^{3}H]proline and [^{14}C]-phenylalanine by *Petunia* pollen after 0, 1, 2 and 3 h of pre-incubation. [^{14}C]phenylalanine, 18.5 kBq·ml^{-1} was added to a final concentration of 0.75 mmol·l^{-1}. The activity of [^{3}H]proline was 370 kBq·ml^{-1}. Proline concentration was as in Fig. 4-2. (—●—●—) uptake of proline; (—○—○—) incorporation of proline; (—▲—▲—) uptake of phenylalanine; (—△—△—) incorporation of phenylalanine.

3.5. *Extent of pool dilution*

The extent of pool dilution can be calculated from the uptake and incorporation rates (Fig. 4-3) in combination with the proline concentrations in the medium and pool (Fig. 4-2). The calculation is only valid if the metabolic conversion of the radioactive compounds into chemically different substances is small. This was checked by 2-dimensional chromatography of the ethanol-soluble pool after prelabeling with [^{14}C]proline and [^{14}C] phenylalanine for 3 h. In these experiments, the radioactivity was recovered at the proline and phenylalanine spots only, which indicates that no significant conversion had occured. Analysis of a hydrolysate of [^{14}C]proline-labeled protein showed that 7% of the count is present in hydroxyproline. The rates of incorporation expressed as counts·min^{-1} in

the four labeling periods were calculated for both amino acids from the regression lines in Fig. 4-3. The calculation of the actual amount of phenylalanine incorporated per unit of time is based on these data and on the specific activity of the amino acid in the medium. The question of whether or not the phenylalanine taken up mixes with the endogenous pool is immaterial, as this pool is very small.

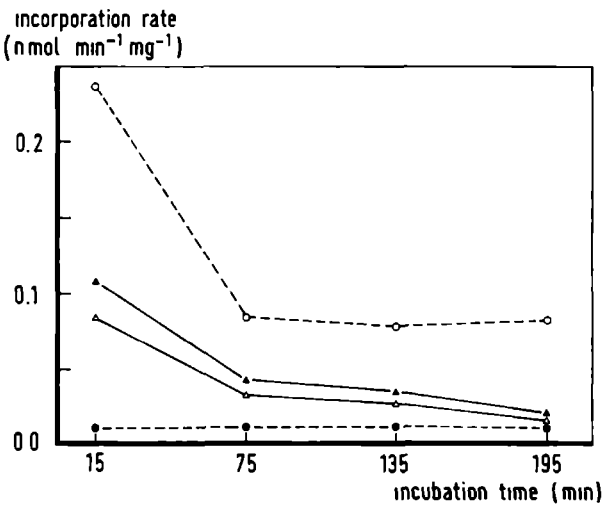

Figure 4-4. Calculated rates of proline and phenylalanine incorporation into proteins. Incorporation rates expressed as counts·min^{-1} were calculated from the regression lines in Fig. 4-3. These data were converted to nmol·min^{-1} on the basis of different assumptions; (--o--o--) proline incorporation calculated from proline count, complete mixing of tracer and endogenous pool prior to incorporation assumed; (--●--●--) proline incorporation calculated from proline count, no dilution by endogenous proline assumed; (—▲——▲—) proline incorporation calculated from phenylalanine count and ratio of proline plus hydroxyproline versus phenylalanine in proteins; (—△——△—) phenylalanine incorporation.
For details, see text.

Three different calculations were made for the actual incorporation rate of proline. The first calculation is based on the ^{3}H-count incorporated and the average specific activity of proline in the pollen. In this case, complete mixing of labeled proline and endogenous pool is assumed. In the second calculation, the rate of proline incorporation is calculated from the ^{3}H-count and the specific activity of proline in the medium. In this case, it is assumed that only proline taken up from the medium is used for protein synthesis, the endogenous pool being completely inactive in this respect. The third calculation is based on the incorporation rate of phenylalanine and on the ratio of proline plus hydroxyproline versus phenylalanine in the proteins. It is seen (Fig. 4-4) that the result of the third approach is intermediate between the two others. The extent of pool dilution can be estimated by comparing the specific activity of proline in the medium with its calculated specific activity (third calculation above) in the newly synthesized proteins. The dilution ratios are 11.0, 3.9, 2.6, 1.9 in the first, second, third, and fourth hour, respectively. From a comparison of the specific activities in the total cellular pool and in the newly synthesized proteins, it is inferred that maximally 50% of the endogenous proline is available for protein synthesis. This figure may be too high as will be discussed below.

4. DISCUSSION

During development, a tremendous amount of free proline is accumulated in *Petunia* pollen, the concentration of which can amount to more than 200 $mmol \cdot l^{-1}$. Most of the proline is released at the beginning of germination. Although the pool of free proline left in germinated pollen is still large, new proline is synthesized during the first hour after germination. Part of the proline released is reutilized by pollen. An estimate of the total uptake is 60 $nmol \cdot mg^{-1}$ in 3.5 h. It would be interesting to know whether excretion and reabsorption of proline also occur *in vivo* during pollen tube elongation in stigma and style. Under the conditions described, the rate of protein synthesis in the first hour as estimated from phenylalanine incorporation is 2.5 times higher than in the next 3 h. The total amount of protein in pollen increases during early germination

(Fig. 4-2). The newly synthesized proteins might play a role in the initiation of germination and tube elongation.

The conclusion that maximally 50% of the proline pool in the cell is available for protein synthesis is based on the implicit assumption that all the proline taken up from the medium can serve as protein precursor. A second possibility, however, is that the radioactive proline penetrates into different pools only one of which is used for protein synthesis. In this case, the observed pool dilution of the precursor proline could involve a much smaller fraction of the endogenous pool than the calculated 50%.

Evidence in favor of the second possibility comes form the linearity of incorporation kinetics. The pattern suggests a constant specific activity in the proline pool used for protein synthesis which requires a rapid saturation with the isotope upon incubation. This, in turn, would indicate that the actual precursor pool is small and certainly less than 50%. Only a direct determination of the specific activity of precursor proline, e.g., in the peptidyl-tRNA pool (Ilan & Singer, 1975), would enable us to discriminate between these possibilities.

Compartmentalization of amino acid and pools has been reported for a number of plant (Oaks, 1965; Holleman & Key, 1967) and animal (Mortimore et al., 1972; Airhart et al., 1974; Ilan & Singer, 1975; Martin et al., 1977 and McKee et al., 1978) systems. In the most extreme situation, exogenously supplied amino acid is directly incorporated without any dilution (Rosenberg et al., 1963; Hider et al, 1971; Van Venrooij et al., 1974). Because of the absence of an endogenous pool, the same holds true for phenylalanine in *Petunia* pollen.

CHAPTER 5

PROTECTION OF POLLEN GERMINATION FROM ADVERSE TEMPERATURES

PART (1)

A POSSIBLE ROLE FOR PROLINE

ABSTRACT

After germination, pollen grains of *Lilium longiflorum* became very sensitive to short periods of heat stress as shown by the greatly reduced germination percentages upon subsequent incubation at the optimal temperature. Addition of proline to the incubation medium made pollen more resistent to heat. It was demonstrated that in a short time a large amount of proline was taken up by the cell. Germination and metabolic functions were completely or partially protected from heat damage by proline treatment. As well, it was shown that proline treatment at least partially protected pollen grains form cold stress. These results suggest that the high proline concentrations found in pollen of many species may confer resistance to germinating pollen grains at unfavourable temperatures thereby enhancing the chances of successful fertilization.

Proline is one of the most abundant amino acids in pollen of many species (Bathurst, 1954; Virtanen & Kari, 1955; Britikov & Musatova, 1964). This amino acid is readily incorporated into proteins (Tupý, 1964; Britikov et al., 1965; Zhang et al., 1982). Following their hydroxylation these proteins may play an important role in elongation of pollen tubes (Dashek & Harwood, 1974; Dashek & Mills, 1981).

Proline has also been found to serve as a substrate for respiration (Britikov et al., 1965) and as a source of nitrogen (Britikov et al., 1970) and other metabolites (Stewart & Boggess, 1978; Zhang & Croes, 1983, b). None of these functions alone, or in combination, can account for the tremendous amount of proline accumulated in pollen. In *Petunia* pollen the proline pool is more than 200 $nmol \cdot mg^{-1}$ but only 5 - 6% of it is used for protein synthesis and respiration during 3 h of *in vitro* incubation (Zhang et al., 1982; Zhang & Croes, 1983, b). Most of the proline is released into the medium. As pollen *in vivo* can presumably get nutriens from the female tissues after pollination, it is unlikely that bulk proline serves as a storage compound.

It is a well-known phenomenon that free proline accumulates in many plants exposed to various stress conditions such as water stress (Barnett & Naylor, 1966; Singh et al., 1972; Blum & Ebercon, 1976), Salinity (Stewart & Lee, 1974; Trelchel, 1975) air pollution (Godzik & Linskens, 1974), and unfavourable temperature (Chu et al., 1974; Chu et al., 1978). This apparent correlation between proline accumulation and environmental stress suggests that proline could have a protective function. Recently, Paleg and his coworkers (1981) demonstrated that a number or solutes, including proline, protected enzymes, isolated from various tissues, from inactivation by heat. A similar protective mechanism might be operating in pollen under adverse conditions. The purpose of the present study was to examine the effect of proline on pollen germination. Lily pollen was selected for this study because the endogenous proline concentration can be manipulated by changing the composition of the incubation medium. A protective function of proline in pollen was demonstrated at unfavourably high and low temperatures.

2. MATERIALS AND METHODS

2.1. Plant material

Fresh anthers were collected in the greenhouse on the day of anthesis from plants of *Lilium longiflorum* (cv. Arai 5) and dried over phosphorus pentoxide in a desiccator overnight. Pollen was brushed off the anthers into a Petri dish, mixed thoroughly and stored at 0 - 4°C for a maximum of 2 days.

2.2. Cold or heat treatment and germination

After equilibration at room temperature, the pollen was swollen in a medium containing sucrose (0.29 mol·l^{-1}), calcium nitrate (1.27 mmol·l^{-1}), boric acid (0.16 mmol·l^{-1}), potassium nitrate (0.99 mmol·l^{-1}; pH 5; Dickinson, 1968), tetracycline (3 mg·l^{-1}), and different concentrations of L-proline or extra sucrose. 6 milligrams of pollen were incubated in 50 µl of medium for 5 min at room temperature. The flask was then placed either in a cold room (0 - 4°C) for 50 h or at 45°C in a water-bath for 10 min. After cold or heat treatment, the culture was diluted 40 times with Dickinson medium and incubated at 27°C for 3 h or 18 h. The control cultures consisted of pollen swollen as described above and then incubated immediately at 27°C. The incubation was terminated and the pollen was stained by adding a few drops of cotton-blue solution (Datta & Naug, 1967). A sample containing at least 1,000 pollen grains was randomly taken, and the germination percentage was determined under the microscope. All pollen grains in the sample were counted and only those which had a pollen tube longer than half the diameter of the grain were considered as germinated.

2.3. Uptake of proline

For determining L-proline uptake by pollen during swelling, 37 kBq·ml^{-1} of L-[U-^{14}C]proline were added to the medium. The pollen was then washed four times at 0°C in 5 ml of unlabelled Dickinson medium. Free proline was extracted in 70% ethanol containing 1% thiodiglycol and 0.07% citric

acid. Aliquots of the extract were mixed with Aqualyte (Baker) and counted in a liquid scintillation analyser (Philips PW-4540). Internal concentrations of free proline were calculated from the uptake and the volumes of the pollen grains as measured according to Gilissen (1977).

2.4. Incorporation of leucine into proteins

The rate of protein synthesis in pollen incubated at 27°C after heat treatment or proline in combination with heat treatment was determined by following the incorporation of L-[^{3}H]leucine into the ethanol-insoluble fraction. L-[^{3}H]leucine, 0.5 mmol·l^{-1}, was added to the culture medium at 370 kBq·ml^{-1}, at different intervals during the 4 h incubation. Following a 20 min pulse-labelling the pollen was washed as described above in cold medium containing 1 mmol·l^{-1} L-leucine and four times with 70% ethanol medium. The ethanol-insoluble fraction was digested in Solulyte (Baker) at 40°C overnight and then mixed with Lipofluor (Baker). All radioactivities measured were corrected for quenching.

2.5. Respiration and amino acid assay

O_2-uptake of pollen incubated in the presence or absence of L-proline was measured manometrically at 27°C (Umbreit et al., 1964).

The amino acids in the free pool were extracted and assayed as described earlier (Zhang et al., 1982).

All experiments were performed at least in duplicate.

2.6. Radiochemicals

L-[U-^{14}C]proline, 8.9 GBq·mmol^{-1}, was a product of ICN Pharmaceuticals. L-[4,5-^{3}H]leucine, 2.5 TBq·mmol^{-1}, was purchased from the Radiochemical Centre, Amersham.

3. RESULTS

3.1. Effect of heat on pollen germination

In an attempt to imitate conditions present in nature we studied the effect of heat on germination by exposing lily pollen after imbibition to supra-optimal, but not too extreme, temperatures, for short periods of time. Germination was then allowed to proceed at 27°C. A treatment at 45°C for 10 min results in a serious drop of the germination percentage 3 h later (Table 5-1-1). The heat effect is partially transient as a considerable portion of the grains start germinating within 18 h.

Table 5-1-1. Effect of heat on germination of lily pollen. After swelling at room temperature for 5 min the pollen grains were exposed to 45°C for 10 min and germinated at 27°C

Heat treatment	Time of incubation at 27°C (h)	Germination (%)
Yes	3	4
	18	40*
No	3	76
	18	80*

* Because of the presence of long pollen tubes the number of germinated grains at 18 h cannot be determined with the same accurary as at 3 h

Proline added to swelling medium is by itself somewhat deleterious as it causes a reduction in germination when added to the non-heated controls (Fig. 5-1-1). However, in the heat-stressed sample it virtually completely reverses the heat effect at the optimal concentration (approximately 0.85 $mol \cdot l^{-1}$). For this sake of comparison, the experiment was repeated with sucrose instead of proline (Fig. 5-1-1) and similar results were ob-

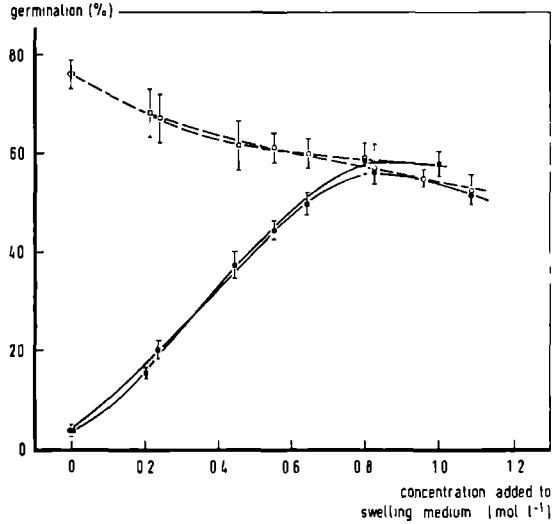

Figure 5-1-1. Germination of lily pollen: the pollen was swollen in Dickinson medium supplemented with different concentrations of L-proline or sucrose and heat-treated at 45°C for 10 min. Afterwards the pollen was incubated in Dickinson medium at 27°C for 3 h; (—●—●—) swollen in proline medium; (—■—■—) in sucrose medium. Pollen without heat treatment: (--o--o--) swollen in proline medium; (--□--□--) in sucrose medium.
Vertical bars indicate standard error (n = 4 for the heat-treated samples, n = 2 for the control).

tained. These data taken together show that proline non-specifically protects pollen germination from heat damage.

3.2. Uptake of proline

For an understanding of the mechanism of proline action in pollen germination knowledge about the uptake of the amino acid is of critical importance. Pollen was swollen in the presence of different concentrations of ^{14}C-labelled proline and the uptake was determined after swelling. A concentration-dependent accumulation resulting in a linear relationship between external and internal proline concentration was observed (Table 5-1-2).

Table 5-1-2. Uptake of [U-^{14}C]proline during swelling for 5 min by lily pollen incubated in Dickinson medium supplemented with different concentrations of proline

Medium	Pollen	
[Proline] (mmol·l^{-1})	Proline uptake (nmol·mg^{-1})	[Proline]* (mmol·l^{-1})
100	105	36
400	371	137
800	643	268
1000	773	336

* Estimated from uptake and calculated volume of pollen

The effect of proline uptake on the size and the composition of the free amino acid pool is shown in Table 5-1-3. Samples were analysed after the 40-fold dilution with Dickinson medium. Swelling and heat treatment in the presence of proline result in an increase of total pool size and proline content by approximately 105 nmol·mg^{-1}. As a consequence, the contribution of proline to the total pool has risen to 60%. This is not unlike the natural situation in *Petunia* pollen where proline is the most abundant amino acid (Table 5-1-3).

3.3. Effect of proline on metabolism of heat-stressed pollen

Oxygen uptake in differently treated pollen cultures was followed as an indication of general metabolism. An advantage of this method is that it is insensitive to proline in non-heated controls even at high concentrations of the amino acid (Fig. 5-1-2-b). Samples of pollen were swollen and heated in media with and without proline and respiration was measured during the subsequent incubation at 27°C (Fig. 5-1-2-a). Respiratory capacity is seen to be severely damaged by heating in the absence of proline.

Table 5-1-3. Effect of proline uptake on the relative abundance of the major components of the free amino acid pool. Samples were analysed 10 min after heat treatment in the absence or presence of proline, 0.85 mol·l^{-1}, except for *Petunia* pollen which was ungerminated

	Lily				*Petunia*	
	- Proline		+ Proline			
amino acid	(nmol·mg^{-1})	(%)	(nmol·mg^{-1})	(%)	(nmol·mg^{-1})	(%)
Thr	7.2	9.9	7.3	4.1	30.2	7.8
Ser	6.9	9.5	7.8	4.4	13.2	3.4
Glu	15.9	22.0	10.3	5.8	43.3	11.1
Pro	1.1	1.5	106.2	59.9	213.9	54.9
Ala	3.3	4.6	7.5	4.2	34.1	8.8
Leu	0.5	0.7	0.4	0.2	0.9	0.2
Arg	18.4	25.4	17.3	9.8	7.8	2.0
Total	72.4	100.0	177.3	100.0	389.6	100.0

Thr = threonine; Ser = serine; Glu = glutamic acid; Pro = proline; Ala = alanine; Leu = Leucine; Arg = Arginine

However, when proline was present at the concentration at which pollen germination is maximally protected (0.85 mol·l^{-1}), the respiration rate was nearly indistinguishable from the control (Fig. 5-1-2-a,b). Lower concentrations of proline have less pronounced effects. Protein synthesis was chosen as a second parameter of metabolism. Pollen was incubated after the heat shock for 4 h and pulse-labelled at intervals with L-[^{3}H]leucine. The incorporation of label in the ethanol-insoluble pool is shown in Fig. 5-1-3. A severe reduction of protein synthesis is brought about by the heat treatment and this effect is only partially overcome by proline. It is interesting to note that in the presence of proline the heat effect is on the initial rate of protein synthesis as later on the same increase is

observed as in the control. In the absence of proline, however, no significant increase with time is found.

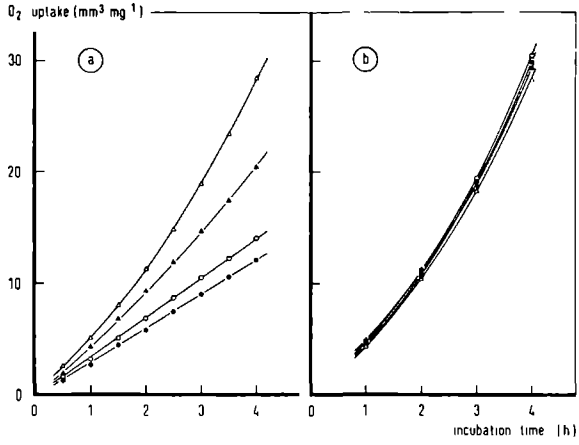

Figure 5-1-2. (a) O_2-uptake by lily pollen at 27°C after heat treatment (45°C for 10 min). Pollen was swollen in Dickinson medium (—●—●—) and in the same medium supplemented with proline: (—○—○—) 245 mmol·l^{-1}; (—▲—▲—) 550 mmol·l^{-1}; (—△—△—) 850 mmol·l^{-1}.
(b) O_2-uptake by lily pollen not given heat treatment. Symbols represent the same proline supplement as given above.

3.4. *Effect of cold on germination*

The question now arises as to whether the protective role of proline is specific with regard to the kind of stress applied. To test this we subjected pollen to cold (0 - 4°C) temperatures for 50 h and screened germination capacity at 27°C following this cold treatment. The pollen grains were placed in medium containing different concentrations of proline. The results show a similar but quantitatively different proline effect from that observed after heating (Fig. 5-1-4). In this case proline can only par-

tially overcome the decline in germination percentage imposed by the adverse temperature.

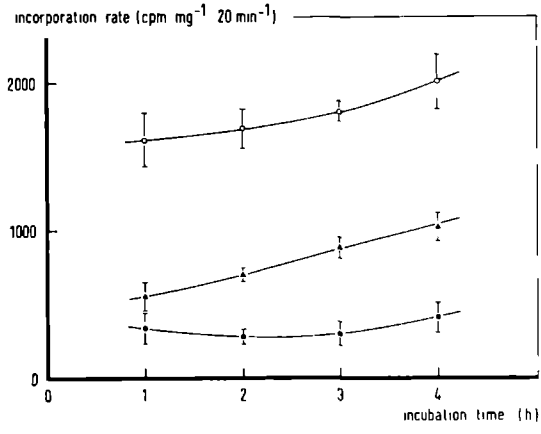

Figure 5-1-3. Protein synthesis of heat-shocked lily pollen. The pollen was incubated at 27°C after heating and pulse-labelled at intervals with [^{3}H]leucine for 20 min. (—●—●—) Swollen in Dickinson medium and heat-treated at 45°C for 10 min; (—▲—▲—) swollen in 0.85 mol·l^{-1} proline medium and heat-treated; (—○—○—) control, no proline, no heat treatment. No difference in the rate of proline uptake were found.
Vertical bars indicate standard error (n = 2).

4. DISCUSSION

Lily pollen becomes sensitive to unfavourable temperatures directly after swelling. The sensitivity is strongly reduced by the presence of proline in the medium during imbibition. Although the effect is not specific for proline, its signifiance lies in the abundance of proline in the pollen of a large number of species (e.g. *Petunia*). It is tempting to speculate that in these species proline protects pollen germination from damage due

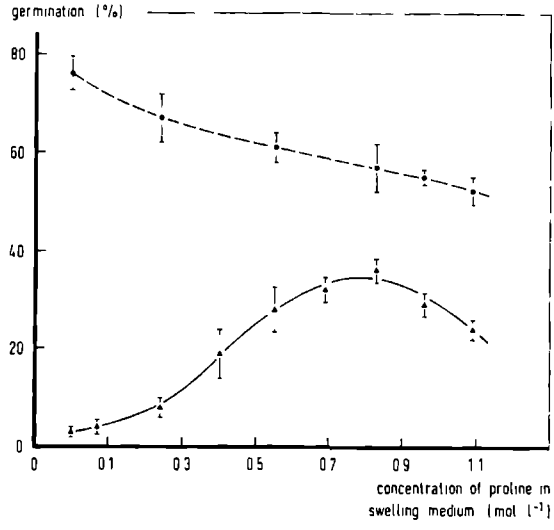

Figure 5-1-4. Germination percentage of lily pollen: the pollen was swollen in Dickinson medium containing different concentrations of L-proline and subjected to cold treatment at 0 - 4°C for 50 h. The pollen was then incubated at 27°C for 3 h (—▲—▲—); control, no cold treatment (—●—●—).
Vertical bars indicate standard error (n = 3 for the cold-treated samples, n = 2 for the control).

to adverse temperatures.

A prerequisite for this hypothesis would be that, in our experimental lily system, a pool of proline accumulates of at least the same order of magnitude as is found in proline-rich pollen. The proline pool accumulated during swelling for 5 min in 0.8 mol·l^{-1} proline medium, at which concentration germination is optimally protected, exceeds the proline pool in *Petunia* by a factor of 3 (Table 5-1-2).

The heat shock applied has a strong impact on cellular metabolism which manifests itself upon subsequent incubation at 27°C. The respiration rate is reduced by 55 - 60% but the initial rate of protein synthesis is even more dramatically affected. Possibly as a result of this difference, pro-

line at 0.85 mol·l^{-1}, while completely eliminating the temperature effect on respiration, only partially reverses the effect on protein synthesis. It is remarkable that despite the low initial rate of protein synthesis in the presence of proline, a germination level of 55% is attained after 3 h of incubation.

Any explanation of the mechanism of the protection by proline should take into account the similarity of the proline action in heat- and cold-stressed pollen, and the fact that dry, ungerminated pollen is completely insensitive to the temperatures applied. Paleg et al. (1981) observed that proline protects several isolated enzymes from heat. They hypothesized that proline might stabilize protein configuration by maintaining the hydration shells around the molecules. The relatively mild conditions of our heat treatment, together with the similarity of the effect of proline on heat- and cold-stressed cells, make it unlikely that this mechanism is the basis of the proline effect in pollen.

Another possibility would be that the massive entrance of proline into the cell caused a drop in the water potential inside and, therefore, a strong reduction in the activity of the cellular water. In this way a kind of dormancy would be imposed creating conditions more or less similar to those in dry, ungerminated pollen. An insensitivity to unfavourable temperatures would be the logical consequence.

Before a more definite answer about the mechanism of proline action can be given, we need a better understanding of the temperature effect itself. There is the added complication that not all the pollen grains react to the heat shock in the same way. Approximately one-half of the cells is rendered unviable, whereas in the other germination is delayed to various degrees.

In nature, abortion of part of the pollen grains or even a serious delay of pollen tube growth would greatly reduce the chances for successful fertilization. Any mechanism that would make germinated pollen more resistant to transient periods of high or low temperature would be an evident selective advantage in many species. Accumulation of a proline pool seems to be such a mechanism.

PART (2)

QUALITATIVE CHANGES IN PROTEIN SYNTHESIS IN GERMINATING POLLEN OF *LILIUM* AFTER HEAT SHOCK

ABSTRACT

After heat shock, protein synthesis is strongly inhibited in germinating pollen of *Lilium longiflorum*. However, the actual extent of inhibition depends on the protein precursor used. It was found that the patterns of newly synthesized proteins obtained from the heat-treated pollen are strikingly different from the control. This dramatic effect can be completely reversed or nearly so by high concentration of proline.

1. INTRODUCTION

In previous studies on the role of proline in pollen germination and tube growth, the amino acid was found to be used for protein synthesis, and to be converted to other molecules whereby part of its carbon moiety is released as CO_2 (Britikov et al., 1965; Zhang & Croes, 1983 b; Zhang et al., 1982). These processes, however, can not account for the enormous proline content of pollen so characteristic for many species (Bathurst, 1954; Britikov & Musatova, 1964). As a consequence, a non-metabolic role was proposed. In lily pollen where proline is not accumulated naturally, artifical loading with the amino acid confers a high resistance to high and low temperatures to imbibed pollen (Zhang & Croes, 1983 a). The effect is also visible at the metabolic level. A heat shock which strongly reduces

pollen respiration in unsupplemented germination medium, is ineffective in this respect in the presence of proline. However, other functions are only partially protected from heat by proline. The rate of protein synthesis, for instance, measured after a heat shock in the presence of proline is much lower than in the untreated control. This is remarkable as germination is not affected under this conditions. The possibility exists that after heating protein synthesis becomes different, not only quantitatively but also qualitatively. The high temperature could have several effects on the composition of the newly-synthesized polypeptides. Specific proteins have been found to appear in response to heating in a number of animal and plant cells (Kelley & Schlesinger, 1978; Sondermeijer & Lubsen, 1978; Vincent & Tanguay, 1979; Barnett et al., 1980). Heat could also change protein composition by affecting the formation of some polypeptides to a greater extent than that of others. If so, these changes would pose interesting questions concerning the relation between protein synthesis on one hand and pollen germination and tube development on the other.

In this paper, we report on profound changes in the composition of the newly-synthesized proteins resulting from a short heat treatment prior to pollen germination.

2. MATERIALS AND METHODS

2.1. Plant material

Fresh pollen of *Lilium longiflorum* (cv. Arai 5) was used in all experiments. The anters were collected from plants grown in the greenhouse and the pollen was gathered and handled as described before (Zhang & Croes, 1983 a).

2.2. Incubation and labeling of pollen

The pollen was swollen, heat-treated and incubated as described previously (Zhang & Croes, 1983 a).

In experiments aimed to quantify uptake and incorporation of amino acids, [^{3}H]leucine, 370 KBq·ml^{-1} and [^{14}C]phenylalanine, 74 KBq·ml^{-1} were added to the medium at 0.5 mmol·l^{-1}. Uptake and incorporation were deter-

mined as described before (Zhang & Croes, 1983 a).

In experiments, in which a very efficient labeling was to be attained, the pollen was collected after heat treatment, gently washed 3 times in 3 ml of germination medium and resuspended in the same medium. [^{3}H]leucine and [^{14}C]phenylalanine were added withoud dilution. The radioactive concentration varied with the sample but the activity of leucine was always 10 times higher than that of phenylalanine.

2.3. Extraction and electrophoresis

After labeling, the pollen was washed 4 times in 5 ml of incubation medium at 0°C and homogenized in 1 ml of NaCl-solution (1 mol·l^{-1}) with a glass homogenizer in an ice-cold water bath. After centrifugation at 18,000*g* for 10 min, the supernatant was mixed with 1 ml of cold acetone and allowed to stand overnight at -5 to -10°C. The NaCl-acetone solution was discarded and the precipitate was taken up in 100 µl of a solution containing 2% sodium dodecyl sulfate (SDS), 5% β-mercapto-ethanol, 10% glycerol and 0.005% bromophenol blue and boiled for 3 min. Electrophoresis on 11% SDS polyacrylamide gels was performed according to Laemmli (1970). The proteins in the gel were visualized by Coomassie blue R-250 staining.

2.4. Autoradiography

After SDS gel electrophoresis, the water in the gel was removed by soaking the gel in dimethylsulphoxide (DMSO) with several changes. 2,5-diphenyl-oxazole (PPO) was then introduced into the gel according to Bonner (1974). After drying, the gel was exposed to a X-ray film (Du Pont Cronex 4) at -70°C for 8 weeks.

2.5. Radiochemicals

L-[4,5-^{3}H]leucine, 2.5 TBq·mmol^{-1}, was purchased from the Radiochemical Centre, Amersham, UK. L-[U-^{14}C]phenylalanine, 16.7 GBq·mmol^{-1}, was obtained from ICN Pharmaceuticals, USA.

3. RESULTS AND DISCUSSION

3.1. Amino acid incorporation in heat-shocked pollen

Germination of lily pollen is very susceptible to heat. Even a moderate rise in temperature (45°C) for a short period (10 min) immediately after imbibition strongly reduces and delays germination (Zhang & Croes, 1983 a). Metabolic processes are also strongly affected. Incorporation of amino acids into proteins when measured after the heat shock is much lower than in the control for a long period of time (Table 5-2-1).

Table 5-2-1. Effect of a heat shock on the subsequent incorporation of leucine and phenylalanine into germinating lily pollen. At the times indicated 1-ml cultures were supplemented with 370 KBq [^{3}H]leucine and 74 KBq [^{14}C]phenylalanine at a final concentration of 0.5 mmol·l^{-1} of both amino acids. Incorporation was measured 0.5 h later

Incubation time (h)	Heat-shocked pollen			Control		
	leu*	phe*	ratio	leu*	phe*	ratio
1	34	105	0.32	161	200	0.81
2	28	102	0.27	169	186	0.91
3	30	150	0.20	180	330	0.55
4	41	216	0.19	201	475	0.42

* in pmol of exogenously supplied amino acid per mg pollen

However, the actual extent of inhibition depends on the protein precursor used. The heat shock reduces the subsequent incorporation of leucine to approximately 20%. In contrast, phenylalanine incorporation is decreased by only 50%. As a consequence, the ratio of externally supplied leucine and phenylanaline fixed in the proteins strongly alteres by the preceding heat shock. This could mean two things. First, the heat treatment might

affect the sizes of the amino acid pools in the cells resulting in different extents of label dilution in heat-treated and control samples. Second, the heat treatment might cause a change in the proportions at which individual proteins are synthesized or even lead to synthesis of new proteins. If such changes would occur, they could be a factor in the severe reduction of germination by the heat shock. The existence of this relation could be tested by comparing the heat effect on the pattern of protein synthesis in the presence and in the absence of a protective proline concentrations. This analysis should take into account a possible direct effect of the high proline concentration on the incorporation of a protein precursor which is unrelated to the temperature treatment. For this reason, the experiments were run in the four possible combinations of the variables: heat-treated pollen in the presence (PH) or absence (DH) of proline; unheated controls in the presence (P) or absence (D) of proline.

3.2. Effect of proline on uptake of leucine

Another factor that must be reckoned with is interference of proline with the uptake of the protein precursor into the pollen grains. This effect was tested by studying the uptake of leucine given at two concentrations to differently treated pollen cultures (Table 5-2-2). Heating as well as proline appear to inhibit leucine uptake at low leucine concentration. In both cases, inhibition is overcome for a great deal by increasing the concentration of leucine. At this level, however, the isotope is too much diluted for the heavy labeling of proteins required for autoradiography of protein bands separated by electrophoresis. To accommodate for this difficulty, we washed the pollen three times with germination medium after the heat shock and adapted the radioactive concentration of the precursor molecule in the fresh incubation medium to the presumed uptake rates of the different samples. In this way sufficiently labeled proteins were obtained.

3.3. Patterns of protein synthesis

The proteins synthesized in heat-shocked and control pollen samples during the 30 min of incubation and separated by polyacrylamide gel electro-

Table 5-2-2. The effect of heat treatment and of proline on the uptake of leucine supplied at two concentrations. Lily pollen was heat-shocked in germination medium with (PH) or without (DH) proline, 0.85 mol·l^{-1}. After resuspension in 40 volumes of germination medium without proline, [^{3}H]leucine was added to a final radioactive concentration of 148 KBq·ml^{-1}. The cultures were analized after a 20-min incubation at 27°C. Controls in medium with (P) or without (D) proline received no heat treatment.

Treatment	Uptake of leucine			
	from 1 µmol·l^{-1}		from 500 µmol·l^{-1}	
	(pmol·mg^{-1})	(%)	(pmol·mg^{-1})	(%)
D	7.6	100.0	696	100.0
P	1.0	13.4	594	85.3
DH	3.4	44.6	555	79.7
PH	0.7	8.8	525	75.4

phoresis are shown in Fig. 5-2-1. [^{14}C]phenylalanine and [^{3}H]leucine were used as labels. In the absence of proline, the patterns obtained from heat-treated pollen and from the control are strikingly different. Only a few bands are lost or decreased in density by heat treatment. On the contrary many proteins appear as new or much denser bands especially in the high molecular weight region of the gel. It may be concluded that normal protein synthesis is completely disturbed as a result of the heat shock. This dramatic effect is completely reversed or nearly so by proline, as its presence during the heat treatment leads to a composition of the newly synthesized proteins which is nearly indistinguishable from the control. This means that proline strongly protects protein synthesis in its qualitative aspects.

Only minor differences are observed between the banding patterns obtained from phenylalanine and leucine labeled pollen. These differences show up in the regions around 63 and 30 k. In the latter portion of the gel one band, at 31 k strongly increases as a result of the heat shock.

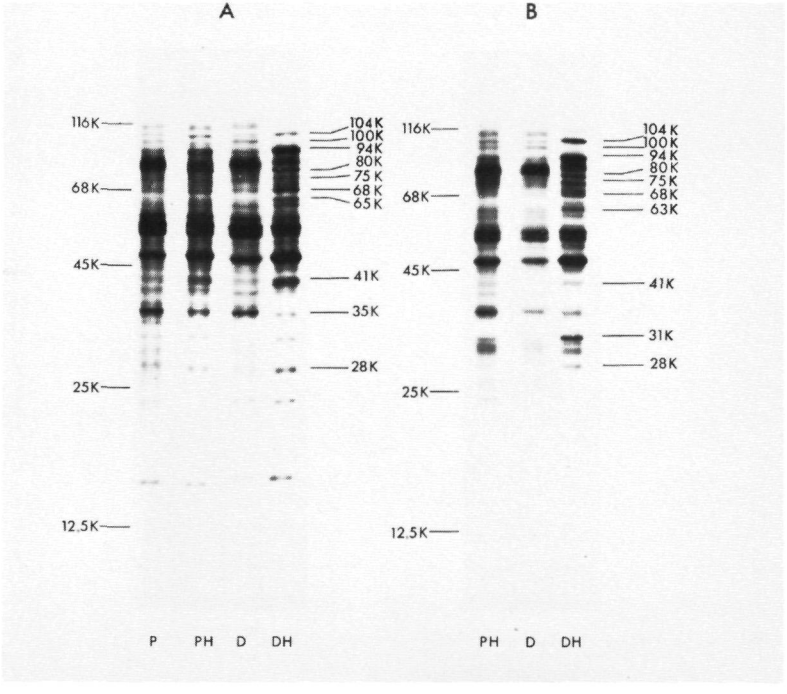

Figure 5-2-1. Autoradiograms of SDS polyacrylamide gels of proteins in germinating pollen of *Lilium longiflorum*. The pollen was heat-treated at 45°C for 10 min in the presence (PH) and in the absence (DH) of proline. Controls with (P) and without (D) proline received no heat treatment. The pollen was then incubated at 27°C and labeled with [^{14}C]phenylalanine (A) or [^{3}H]leucine (B) for 30 min. [^{14}C]phenylalanine was added at 7.4, 444, 11.1 and 158 KBq·ml^{-1} to D, DH, P and PH cultures respectively. In case of [^{3}H] leucine, the radioactivities used were 10 times higher. The molecular weight markers are: β-galactosidase (116K); bovine serum albumin (68K); hen egg albumin (45K); chymotrypsinogen A (25K) and cytochrome C (12.5K).

The effect is reversed by proline. A comparison of the unheated controls incubated with and without proline shows that proline by itself has no effect on the quality of protein synthesis. The spectacular increase in the proportion of the high-molecular weight proteins as a consequence of the heat shock could mean that their synthesis is specifically induced or enchanced by the abnormal conditions as has been described for soya

(Barnett et al., 1980). An alternative would be that the heat treatment leads to a disturbance or post-translational protein modification. As a result of this, high molecular weight precursor molecules would no longer be processed and would accumulate in the cell. If early protein synthesis is required for pollen germination and tube growth, the effect of a temperature shock on protein composition is certainly sufficient to account for the strongly reduced germination percentage. The same holds true for the reverse effects brought about by proline. This would mean that a decrease in the rate of protein synthesis as found in pollen heat-treated in the presence of proline (Zhang & Croes, 1983 a) is in itself not deleterious to germination provided the composition of the end products remains unaltered.

The minor deviations observed in the banding patterns of phenylalanine and leucine-labeled proteins are insufficient to account for the difference in the ratios of incorporation of these two precursors between heat-shocked and control pollen (Table 5-2-1). A quantitative analysis of label distribution in the whole set of proteins is needed to solve this problem. This means that the effects of label dilution by cellular pools on the differential amino acid incorporation can not be excluded at this moment.

CONCLUSION

Accumulation of free proline in pollen grains is a common phenomenon in nature (Britikov, 1975). The level of the accumulation can be as high as 200 nmol·mg^{-1} (Tupý, 1963) or even more (Linskens & Schrauwen, 1969). The present investigation shows that the accumulation of proline proceeds during the entire process of anther development, from the microspore stage untill anthesis, except for an intervening period when the bud length is between 15 and 35 mm (Chapter 2). The rate of proline accumulation is very high, despite the intervening period, and was measured to average about 2.5 µg·d^{-1}·mg dry weight^{-1}.

There are several factors which might be involved in the mechanism of proline accumulation in anthers. Firstly, in the case of *Petunia*, the anther is a sink for proline and a large amount of the amino acid is transported from the leaves. The transportation continues throughout the whole period of development. Calculations show that about 40% of the proline accumulated in the anthers of *Petunia* is transported (Chapter 2).

The second factor which contributes to the proline pool is the synthesis of proline in the anthers. A high ratio of conversion from glutamate to proline was found in *Petunia* anthers in comparison with the other parts of the flower bud. Normally the proline biosynthesis pathway displays end product inhibition, with proline inhibiting the first step of the conversion from glutamate. The continuous synthesis of proline in the presence of a high concentration of this amino acid in the anthers suggests that there is a loss of feedback control. This phenomena has also been reported in water-stressed leaves (Boggess et al., 1976).

Proline accumulation could also be regulated by some growth substances, e.g. ABA, which may stimulate the synthesis of proline (Huber, 1974; Stewart, 1980).

The third factor which influences the size of the proline pool is the rate of drain on this pool. As at least during the first period of anther development large amounts of proteins are synthesized, it is reasonable to expect that a certain amount of free proline will be incorporated into these proteins. Also proline can be utilized in other pathways and some of it might be expected to be oxidized during anther development. The ratio of conversion of proline to glutamate was found to be lower in anthers when compared with other parts of the flower bud (Chapter 2). This indicates that the oxidation rate of proline in anthers is not high. Whether an inhibition of proline oxidation exists in anthers as it does in water-stressed leaves (Stewart & Boggess, 1978) and mitochondria (Sells & Koeppe, 1981) should be investigated further. It is clear that the total amount of proline drained from the free pool is far less than that contributed by transportation and synthesis. This accounts for the large accumulation of this amino acid.

The mechanism of proline accumulation in anthers does not seem to be the same as that found in water-stressed plants, which has been investigated extensively (for references see Aspinall & Paleg, 1981; Stewart, 1981). First of all, transportation is not involved in the latter case. Secondly, in wilted plants it has been shown that protein synthesis is inhibited (Stewart, 1972; Stewart et al., 1977) and suggested that proteolysis is stimulated (Thompson et al., 1966). In view of the net increase in protein content observed during anther development, it is most unlikely that either of the above two factors contributes to accumulation of proline in anthers. Finally, proline accumulation in anthers, at least in the first period of development, does not respond to environmental changes as it does in water-stressed plants. The accumulation regulated by developmental events depend on the significant difference between formation and consumption of this amino acid. The factors which may contribute to it are summarized in Fig. 6-1.

The proline accumulated in pollen may be involved in a number of functions. As it is one of the amino acids which is utilized in proteins, the major metabolic fate of proline normally is to be incorporated

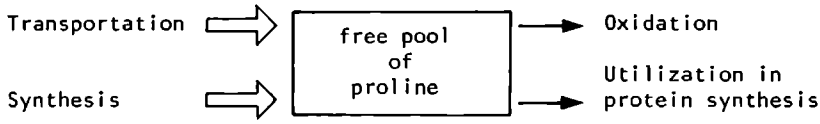

Figure 6-1. The various metabolic processes which result in accumulation of free proline in anthers. Thick arrows indicate processes that occur at a relatively high rate and the thin arrows indicate those processes that occur at a relatively low rate.

into proteins. In pollen of *Petunia*, labeling experiments show that part of the accumulated proline is used for protein synthesis (Chapter 4). A portion of the proline incorporated is further hydroxylated. The newly synthesized proteins may play an important role in pollen germination and tube growth (Chapter 5; Dashek & Harwood, 1974). In discussing incorporation of proline into proteins, attention must be paid to the fact that the free proline pool in pollen may be compartmentalized and the precursor pool for protein synthesis is possibly much less than 50% of of the total proline pool. This implys that quantitatively only a small portion of the accumulated proline is used for synthesis of proteins (Chapter 4).

Proline can be oxidized *via* glutamate and the citric acid cycle to CO_2 and other compounds. In germinating pollen of *Petunia* (Chapter 3), part of the free proline is used as a substrate for respiration. Despite its abundance, the proline is only a minor substrate for this respiration. Even in a culture containing no exogenous metabolizable carbon source, the maximum contribution of proline carbon to total respiration amounts to no more than 1 - 2%. Of the proline C-atoms entering the citrate cycle about 60 - 70% presumably leave in the form of one or more C_4 compounds. They can be in turn used for macromolecular synthesis. Indeed the possibility exists that after oxidation, proline converts to glutamate and the amino group is transaminated, and can be further used for synthesis of amino acids and other nitrogen-containing compounds. As during the period of pollen incubation no decrease in total free proline content was observed (Chapter 4), in combination with the fact that proline is only a minor substrate for respiration, it can be concluded that the amino group from proline is not an important nitrogen source for germinating pollen.

Since pollen and pollen tubes *in vivo* can be presumably get nutrients from the female tissues after pollination (Linskens & Esser, 1959; Labarca & Loewus, 1972; Campbell & Ascher, 1975), it is unlikely that bulk proline serves as a storage compound, although it can play various metabolic functions. Properties of proline itself implicate a possibly protective function. Proline in solution has been shown to affect the solubility of various proteins and to protect bovine serum albumin from denaturation by $(NH_4)_2SO_4$ or ethanol (Schobert & Tschesche, 1978). It is considered to be one of the 'compatible solutes' (Brown & Simpson, 1972). *In vitro* activity of several enzymes (Stewart & Lee, 1974) and respiration capacity (Chapter 5) are completely unaffected by proline even at high concentrations. Proline can also protect enzymes from the deleterious effects of biological toxic compounds like urea (Yancey & Somero, 1979) and protect enzymes from heat inactivation (Paleg et al., 1981). Possessing a low molecular weight and very great solubility, proline is considered to be an osmotic factor which keeps the cell in a more hydrated state. This may be very important for the survival of pollen grains and in maintaining germination capacity after anthesis. In the present studies it has been shown that proline in high concentrations protect pollen germination from adverse low and high temperatures (Chapter 5). After imbibition preceding germination, pollen grains of *Lilium* become very sensitive to even short periods of heating as shown by a severe decrease in germination capacity. Addition of proline to the incubation medium makes pollen more resistant to heat. At the optimum concentrations, proline completely eliminates the temperature effect on respiration and partially reverse the effect on protein synthesis. Furthermore, the heat shock not only depresses the rate of protein synthesis but probably also completely inhibits the synthesis of some special proteins which are very important to pollen germination. In the presence of proline at high concentrations, the synthetic function for these proteins is protected from heat damage. Although the effect of proline is not specific, its significance lies in the abundance of proline in the pollen of a large number of species. In general, the high proline concentration in pollen may confer resistance to the pollen grains at unfavorable conditions thereby enhancing the chance of succesful fertilization. This suggests that accumulation of proline in pollen may be of evolutionary significance.

As a summary, the possible functions of proline in pollen are illustrated in Fig. 6-2. The results of the present study indicate that the non-

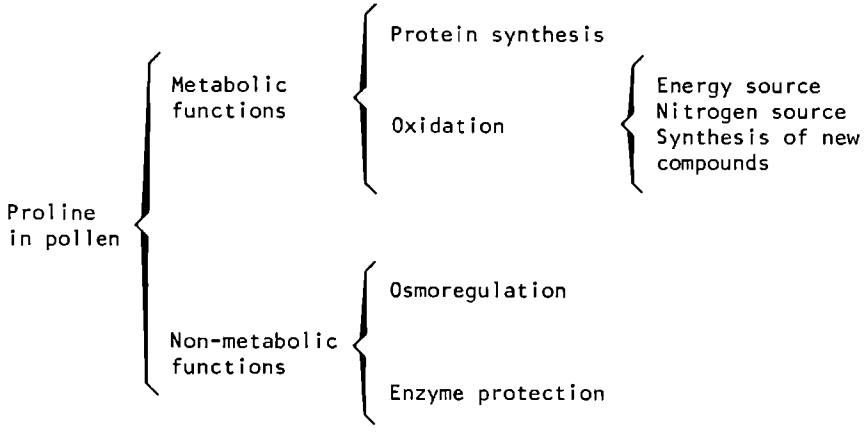

Figure 6-2. The possible functions of proline accumulated in pollen grains.

metabolic functions are the most important functions prior to pollination. A question on proline metabolism not considered in this thesis concerns the functioning and disappearance of the proline pool after pollination. Research into this problem can now be carried out by the use of pollen loaded with radioactive proline during flower bud development.

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De ophoping van vrij proline in pollenkorrels is een in de natuur veel voorkomend verschijnsel (Britikov, 1975). De omvang van deze ophoping kan 200 nmol·mg^{-1} bedragen (Tupý, 1963) of zelfs meer (Linskens en Schrauwen, 1969). Deze proline-ophoping vindt plaats gedurende het gehele proces van de ontwikkeling van de anthere vanaf het microspore-stadium tot de anthese met uitzondering van een tussenperiode, wanneer de lengte van de knop 15 tot 35 mm is. De snelheid van de proline-ophoping is zeer hoog. Ondanks de stagnatie in de tussenperiode is de snelheid gemiddeld 2,5 µg·d^{-1}·mg^{-1} drooggewicht (Hoofdstuk 2).

Verschillende factoren spelen een rol in het mechanisme van de proline-ophoping in antheren. Ten eerste is de anthere, in het geval van *Petunia*, een "sink" voor proline; een grote hoeveelheid van het aminozuur wordt aangevoerd vanuit de bladeren. Het transport verloopt gedurende de gehele periode van ontwikkeling. Een berekening laat zien, dat ongeveer 40% van het proline, opgehoopt in de antheren van *Petunia*, is getransporteerd.

Een tweede factor is de synthese van proline in de antheren. Een hoge verhouding van omzetting van glutamaat naar proline werd bij *Petunia* gevonden in de antheren, in verhouding tot die in de andere delen van de bloemknop. De verklaring voor de voortgezette synthese van proline in de aanwezigheid van een hoge concentratie van dit aminozuur zou kunnen liggen in het mechanisme, dat ontdekt is in bladeren bij watertekort, namelijk dat er een verlies aan terugkoppeling van de synthese door proline optreedt (Boggess et al., 1976). Proline-ophoping zou ook gereguleerd kunnen worden door sommige groeistoffen zoals ABA, die de synthese van proline zouden kunnen stimuleren (Huber, 1974; Stewart, 1980).

Een derde factor is de snelheid van verdwijnen van proline uit zijn pool. Daar tenminste in de eerste periode van de ontwikkeling van de anthere grote hoeveelheden eiwit worden gesynthetiseerd, is het redelijk te veronderstellen, dat een zekere hoeveelheid vrij proline zal worden ingebouwd. Proline is een reactief aminozuur, dat zeker voor een deel zal worden geoxydeerd tijdens de ontwikkeling van de anthere. De omzettingsverhouding van proline naar glutamaat blijkt in antheren laag te zijn in vergelijking met die in andere bloemdelen. Dit houdt in, dat de snelheid van de proline-oxydatie niet hoog is. Of oxydatie van proline in antheren

wordt geremd, zoals in bladeren bij watertekort (Stewart en Boggess, 1978) en mitochondriën (Sells en Koeppe, 1981), moet verder worden onderzocht. In ieder geval is de totale hoeveelheid proline, die de vrije pool verlaat, niet vergelijkbaar met wat wordt getransporteerd en gesynthetiseerd. Anders zou geen ophoping worden verwacht.

Het mechanisme van de proline-ophoping in antheren schijnt niet hetzelfde te zijn als dat in planten bij watertekort, dat uitvoerig is onderzocht (*zie Aspinall en Paleg, 1981; Stewart, 1981*). Ten eerste speelt in het laatste geval transport geen rol. Ten tweede is in verwelkte planten de eiwitsynthese geremd (Stewart, 1972; Stewart et al., 1977) en mogelijk wordt de proteolyse gestimuleerd (Thompson et al., 1966). Hoogstwaarschijnlijk draagt geen van deze beide factoren bij aan de ophoping van proline in antheren. Dit is eenvoudig zo vanwege de netto toename van het eiwitgehalte tijdens de periode van ontwikkeling van de antheren. Tenslotte reageert de proline-ophoping tenminste in de eerste ontwikkelingsperiode niet op veranderingen in de omgeving, zoals dat geschiedt in bladeren van planten bij watertekort; waarschijnlijk wordt het proces in de antheren geregulеerd door de ontwikkeling zelf.

Om het eenvoudig te zeggen: de ophoping van proline in antheren hangt af van het beduidende verschil tussen vorming en gebruik van dit aminozuur. De factoren, die hieraan kunnen bijdragen, zijn samengevat in Fig. 6-1

Figuur 6-1. De verschillende metabolische processen, die leiden tot ophoping van vrij proline in antheren: de dikke pijlen duiden een hoge snelheid aan; de dunne pijlen duiden een relatief lage snelheid aan.

De functie van het proline opgehoopt in pollen kan een aantal aspecten behelzen. Als één van de aminozuren in eiwitten is de belangrijkste metabolische bestemming van proline op een normaal concentratie-niveau, ingebouwd

te worden in eiwitten. Markerings-experimenten (Hoofdstuk 4) laten zien, dat in pollen van *Petunia* een deel van het opgehoopte proline wordt gebruikt voor eiwitsynthese. Een bepaalde fractie van het ingebouwde proline wordt verder gehydroxyleerd. De nieuw-gevormde eiwitten zouden een belangrijke rol kunnen spelen bij de kieming van pollen en de groei van de pollenbuis (Hoofdstuk 5; Dashek en Harwood, 1974). Bij de bespreking van de inbouw van proline in eiwitten moet aandacht worden geschonken aan het *feit, dat de vrije proline pool in pollen gecompartimentaliseerd kan zijn*; de precursor pool voor eiwitsynthese is waarschijnlijk veel kleiner dan 50% van de totale proline pool. Dit houdt in, dat kwantitatief slechts een klein deel van het opgehoopte proline voor eiwitsynthese wordt gebruikt.

Proline is een reactief aminozuur en kan via glutamaat en de citroenzuurcyclus worden geoxydeerd tot CO_2 en andere verbindingen. In kiemend pollen van *Petunia* (hoofdstuk 3) wordt een deel van het vrije proline gebruikt als ademhalingssubstraat. Ondanks de overvloed, waarin het voorkomt, is proline slechts een onbelangrijk substraat. Zelfs in een cultuur, die geen metaboliseerbare koolstofbron in het medium bevat, is de maximale bijdrage van proline-koolstof aan de ademhaling niet meer dan 1 tot 2%. Van de koolstofatomen in proline, die in de citroenzuurcyclus terecht komen, gaat waarschijnlijk 60 tot 70% er weer uit in de vorm van een of meer C_4-verbindingen. Op hun beurt kunnen deze gebruikt worden voor de synthese van macromoleculen. Inderdaad bestaat de mogelijkheid, dat na oxydatie proline wordt omgezet in glutamaat en dat de aminogroep wordt getransamineerd en verder gebruikt voor de synthese van aminozuren en andere stikstofhoudende verbindingen. Daar gedurende de periode van de incubatie van het pollen geen afname van het totale prolinegehalte werd waargenomen, en proline slechts een onbelangrijk ademhalingssubstraat is, kan niet worden verwacht, dat de aminogroep van proline een belangrijke stikstofbron is voor kiemend pollen. Omdat pollen en pollenbuizen *in vivo* waarschijnlijk voedingsstoffen kunnen krijgen uit de vrouwelijke weefsels (Linskens en Esser, 1959; Labarca en Loewus, 1972, 1973; Campbell en Ascher, 1975), is het onwaarschijnlijk, dat het leeuwedeel van de proline dient als reservemateriaal, hoewel proline zelf verschillende metabolische functies kan hebben.

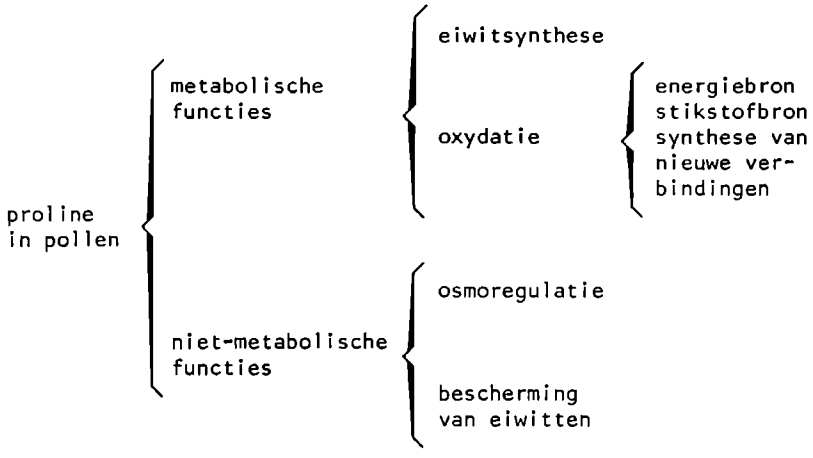
De eigenschappen van het proline zelf houden de mogelijkheid in van een beschermende functie. Proline in oplossing beïnvloedt de oplosbaarheid van verschillende eiwitten en beschermt runderalbumine tegen denaturatie door

ammoniumsulfaat en ethanol (Schobert en Tschesche, 1978). Het is een van de "compatibele opgeloste stoffen" (Brown en Simpson, 1972). De *in vitro* activiteit van verschillende enzymen (Stewart en Lee, 1974) en de ademhalingscapaciteit (Hoofdstuk 5) worden absoluut niet beïnvloed door proline, zelfs niet in hoge concentraties. Proline kan enzymen beschermen tegen biologische giftige stoffen als ureum (Yancey en Somero, 1979) en tegen inactivering door hoge temperatuur (Paleg et al., 1981). Omdat proline een laag moleculair gewicht en een hoge oplosbaarheid bezit, wordt het beschouwd als een osmotische factor, die de cel in een betere hydratie toestand houdt. Dit zou belangrijk kunnen zijn voor de overleving van pollenkorrels en de handhaving van de kiemingscapaciteit na anthese.

Inderdaad beschermen hoge concentraties proline de pollenkieming tegen de nadelige effecten van hoge en lage temperatuur (Hoofdstuk 5). Na de imbibitie, die vooraf gaat aan de kieming, worden pollenkorrels van *Lilium* zeer gevoelig voor zelfs een kort verblijf bij hogere temperatuur, zoals blijkt uit verlies van een belangrijk deel van de kiemingscapaciteit. Toevoeging van proline aan het incubatiemedium maakt pollen resistenter tegen warmte. Bij de optimale concentratie heft proline het temperatuureffect op de ademhaling volledig op en het verkleint het effect op de eiwitsynthese. Voorts onderdrukt de temperatuurschok niet alleen sterk de snelheid van de eiwitsynthese, maar remt ook, waarschijnlijk volledig, de synthese van enkele individuele eiwitten, die zeer belangrijk zijn voor de pollenkieming. In de aanwezigheid van hoge concentraties proline wordt de synthetische functie voor deze eiwitten beschermd tegen beschadiging door warmte. Hoewel het effect van proline niet specifiek is, ligt de betekenis er van in de overvloed aan proline in het pollen van een groot aantal soorten. Dit wijst er op, dat ophoping van proline mogelijk een evolutionaire betekenis heeft. In het algemeen zal de hoge proline-concentratie in pollen de pollenkorrels weerstand geven onder ongunstige omstandigheden en daardoor de kans op succesrijke bevruchting verhogen.

Als samenvatting zijn de mogelijke functies van proline in pollen weergegeven in Fig. 6-2.

Een deel van het proline-metabolisme, dat niet aan de orde is gekomen in dit proefschrift, is het functioneren en verdwijnen van de proline pool na de bestuiving. Onderzoek in deze richting kan nu uitgevoerd worden door



Figuur 6-2. De mogelijke functies van proline, opgehoopt in pollenkorrels.

gebruik te maken van pollen, dat tijdens de bloemontwikkeling gemarkeerd is met radioactief proline.

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CURRICULUM VITAE

Zhang Hong-gi was born on the 6th of May, 1943 in Hebei, China. After studying for 12 years in primary and secondary schools in Beijing (Peking), he enrolled in the Department of Biology, Section of Plant Physiology, Peking University, Beijing in 1962. Following graduation in 1968 he was assigned to Ji-Ze county, Hebei province, to work in a pump factory. Three years later he became a teacher at a secondary school in the same county. In 1978 he returned to Peking University as a postgraduate student in the laboratory of Prof. Hu Shi-yi in the Department of Biology. He passed the examination for going abroad under the auspices of the Ministry of Education of the Peoples's Republic of China and in August 1979 began studies at the University of Nijmegen in the Netherlands. After a short period of studying English he joined the Department of Botany at this university. In April 1980 under the guidance of Prof.Dr. H.F. Linskens and Dr. A.F. Croes, he started a research program on the accumulation and function of proline in pollen. The experimental work for this Ph.D. thesis was completed by the end of May, 1983.

Marriage: In 1969 he married Li Yi-qin. They have two daughters, Zhang Fan and Zhang Hui.

PROPOSITIONS

1. Unscheduled DNA synthesis in *Petunia* pollen is induced by metal ions.
(Jackson, J.F. and Linskens, H.F., *Mol. Gen. Genet.* 187, 112-115, 1982)

2. The winged bean (*Psophocarpus tetragonolobus*, L.) is an important new crop for the tropical region.
(*Winged bean, an annotated bibliography*. International Documentation Center, AIBA/SEARCA, College, Laguna 3720, Philippines, 1981)

3. Wall-bound proteins of pollen tubes play a role in the incompatibility reaction after self-pollination of incompatible *Lilium longiflorum*.
(Li, Y.Q. and Linskens, H.F., *Theor. Appl. Genet.* 67, 1983, in press)

4. In concentration determination of some proteins, such as membrane proteins, the Lowry method with bovine serum albumin (BSA) as a standard is not reliable. A better way is to replace BSA by a standard of the same kind of material from which the protein content has to be determined, as was originally proposed by Lowry *et al.*
(Peters, W.H.M., Fleuren-Jakobs, A.M.M., Kamps, K.M.P., de Pont, J.J.H.H.M. and Bonting, S.L., *Anal. Biochem.* 124, 349-352, 1982; Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J., *J. Biol. Chem.* 193, 265-275

5. Microtubules do not control microfibril orientation in the cell wall of *Equisetum hyemale* root hairs.
(Emons, A.M.C., *Protoplasma*, 113, 85-87, 1982;

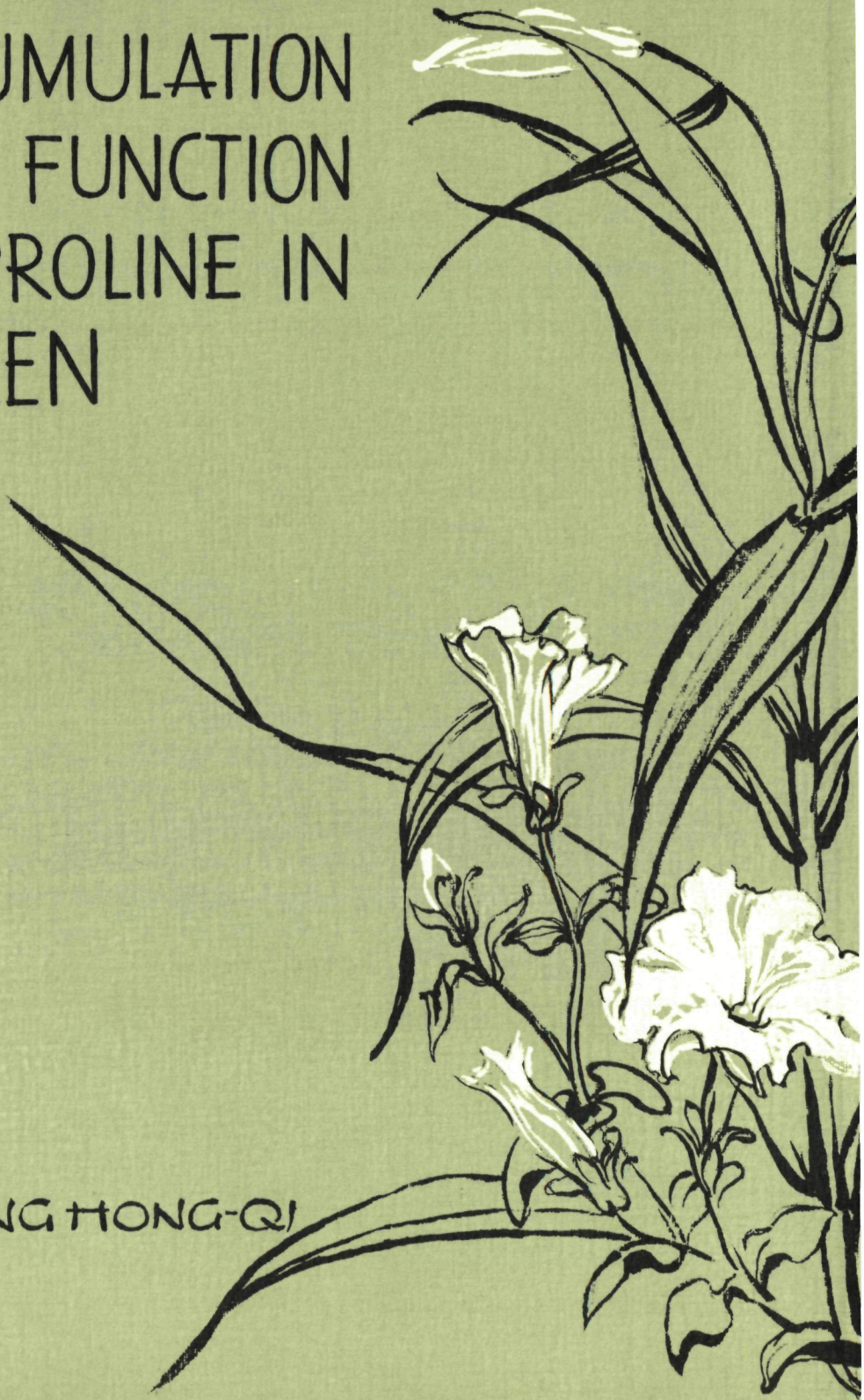
Emons, A.M.C. and Wolters-Arts, A.M.C., *Protoplasma*, 117, 68-81, 1983)

6. The role of the individual hormones in the initiation of flower buds in tissue culture of tobacco challenges the long-held concept that the auxin/cytokinin balance rather than the absolute hormone concentrations is the determining factor in bud regeneration.

7. The gametic fusion which results in the transmission of male gamete nuclei with co-transmission of the sperm organelles in the megagametophyte is not random in *Plumbago zeylanica*.
(Russell, S.T., *Am. J. Bot.* 70, 416-434, 1983)

8. The time course of protein synthesis during imbibition, established using isolated embryos (e.g. Marsh, L., Datta, K. and Marcus, A., *Plant Physiol.* 70, 67-73, 1982) is not likely to reflect the time course under natural conditions.

ACCUMULATION AND FUNCTION OF PROLINE IN POLLEN



ZHANG HONG-QI